

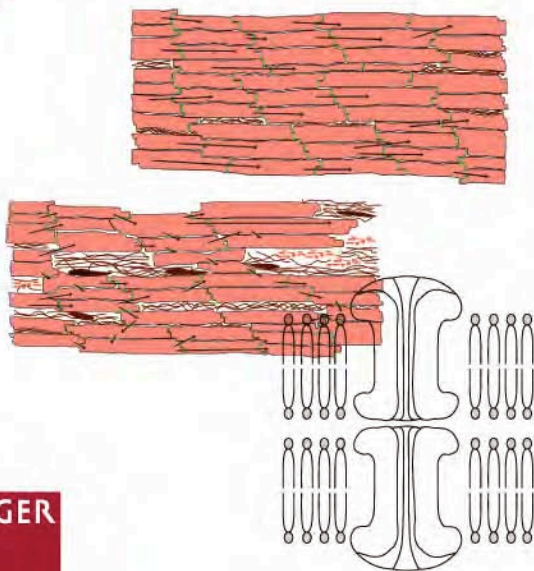
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Vol. 42

# Cardiovascular Gap Junctions

Editor  
**S. Dhein**



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## **Cardiovascular Gap Junctions**

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# Cardiovascular Gap Junctions

Volume Editor

*Stefan Dhein* Leipzig

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## Advances in Cardiology

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## Preface

... all the things do depend upon the motional pulsation of the heart:  
To the heart is the beginning of life.

*William Harvey, 1653*

With this sentence and his investigations, William Harvey started modern cardiovascular research and medicine. While he was fascinated by mechanical motion and discovered a basic principle of the cardiovascular system, recent research focuses on motion itself and its regulation. The expression ‘motional pulsation’ somehow includes rhythm. The basis for this rhythmical motion is an electrical activation wave leading to contraction, which has to propagate from its origin at the sinus node to the whole heart. Thus ‘motional pulsation’ encompasses another form of *motion*, a propagating electrical wave. This is made possible by a network of communicating cells interconnected by gap junction channels. However, these channels not only allow the transfer of electrical signals, they also enable the transfer of small molecules which may serve as signals for cell growth, death or differentiation. In addition, it has become clear that these gap junction channels also importantly contribute to vascular *motion*. It was the classic paper by N.B. Gilula (1944–2000), published in *Nature* [Gilula et al., *Nature*, 1972;235:262–265], that provided the first clear evidence that gap junctions are involved in exchange of metabolites and ions between neighboring cells. Thereafter, our knowledge about these channels and their functions has been enlarged by many elegant studies, and gap junction research has become a focus in cell biology, since intercellular gap junction communication provides the basis of the organization of many organs as a cellular network.



With these studies, it became increasingly evident that gap junctions significantly contribute to the regulation of the cardiovascular system and that failure or alterations of these channels lead to dysfunction.

Since the publication of *Cardiac Gap Junctions* in 1998 [Dhein S, Cardiac Gap Junctions. Physiology, Regulation, Pathophysiology and Pharmacology. Basel, Karger] gap junction research in the cardiovascular system has considerably grown and has largely improved our understanding of the regulation of the heart and vasculature in health and disease. Several well-known gap junction researchers in the cardiovascular field have contributed to the present edition of this book, which is intended to give insight into this fascinating field. I wish to thank them all for their help and support, as well as all the other gap junction researchers who made these and my own studies possible with their basic findings and seminal papers.

The first part of this book is focused on the major aspects of these intercellular channels, allowing the readers who are not familiar with the field to get a deeper understanding of gap junction physiology, pharmacology and regulation, while the second part elucidates their role in the pathophysiology of a number of important cardiovascular diseases, such as arrhythmia, heart failure, ischemia, atrial fibrillation, diabetes and arteriosclerosis. Hopefully, this book will help stimulate researchers to extend their investigations in this fascinating field, and exchange their views and findings in an open communicating scientific network, as the cells they are investigating do.

*Stefan Dhein*  
Leipzig, October 2005

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## Cardiac Connexins: Genes to Nexus

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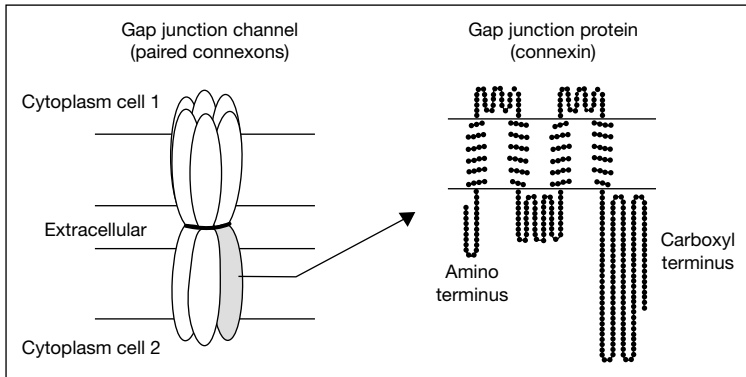
### Abstract

Gap junctions are formed of at least 20 connexin proteins in mammals and possibly pannexins as well. Of the connexins, at least 5 (Cx30.2, Cx37, Cx40, Cx43 and Cx45) are prominently expressed in the heart and each shows regional and cell type specific expression. Contributions of each of these connexins to heart function has been in many cases illuminated by connexin null mice. The cardiac connexin genes whose genomic organization and transcriptional controls have been studied most thoroughly indicate more complex possibilities for alternate promoter usage than originally thought as well a multiple transcription factor binding sites; presumably, such complexity governs developmental timing and regional connexin expression patterns. The structure of cardiac connexin proteins indicate four primarily  $\alpha$ -helical transmembrane domains, cytoplasmic amino and carboxyl termini and a cytoplasmic loop, all of which contain some regions of  $\alpha$ -helix, and extracellular loops that are primarily  $\beta$ -structure. A number of proteins that bind to cardiac connexins are known, and more are certain to be discovered, linking the connexin into an intercellular signaling complex, the nexus. Binding sites may either correspond to structured regions within the connexin molecules or be unstructured, leading to presumably low-affinity and dynamic interactions.

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Gap junctions in adult heart are found at the intercalated disk, localized between the desmosomes and adherens junctions and in small amounts along the lateral myocyte membranes. They are thought to function primarily in passage of electrical impulses from myocyte to myocyte, aiding the synchronous electrical activity of the myocardium. As with desmosomes and adherens junctions, mammalian gap junctions are randomly localized around the myocytes at birth but unlike the other junctional types they remain more scattered during



**Fig. 1.** Schematic of Cx43 based on the PORTER sequence program. All connexins have four transmembrane domains and an intracellular amino and carboxyl terminus. Connexins form oligomers called connexons (or hemichannels) which are the active channel unit.

early postnatal development. As the myocytes develop, the adherens junctional proteins and desmosomal proteins migrate to the longitudinal ends of the cells and form junctional complexes. Following formation of the structural intercalated disk, the gap junction proteins coalesce at these sites, thereby forming the low resistance plaques of gap junction channels between cells [1]. The anisotropic arrangement of gap junctions at the intercalated disks favors the passage of current along the longitudinal axis of the cell, an event underlying the synchronous contraction of the myocardium.

Mammalian genomes contain numerous gap junction proteins (about twenty connexins [2] and three pannexins [3], all with similar topology) (fig. 1). Although little is currently known regarding a possible contribution of pannexins, which possess no sequence similarity to connexins but are weakly homologous to the innexins that form invertebrate gap junctions [4], cardiac connexin distribution and function have been actively studied. Each compartment of the heart contains different connexins, in differing ratios. Each of these connexins has different properties, and each is differentially regulated. While passage of current is a uniform property of channels formed from connexins, unitary conductance and potassium permeability, the major factors dictating the amount of current passed through each gap junction channel, are isoform specific. In this review we begin by discussing the localization of each of the cardiac connexins, including their developmental profile, and what is known regarding their regulation.

## Cardiac Connexins

### *Connexin30.2*

The presence in heart of a novel connexin (mCx30.2 and its human ortholog, hCx31.9) has recently been reported in intercalated disks, as well as in vascular smooth muscle and testis [5–8]. Detailed study of the distribution of mCx30.2 within adult myocardium indicates that its expression is confined to the conduction system, being present in sinoatrial and atrioventricular nodes as well as the atrioventricular conduction bundle. mCx30.2 is absent in working myocardium of atrium and ventricles [6]. Cx30.2 expression thus overlaps that of Cx45 (see below), and may be present in cardiac regions adjacent to Cx40. Notably, exogenous expression of the cardiac connexins in HeLa cells indicates that Cx30.2 forms functionally rectifying channels when paired with Cx40, Cx43 and Cx45, offering the possibility that such pairing could favor unidirectional conduction [6, 7].

The channels formed by the Cx30.2 isoform have miniscule single channel conductance (around 10 pS), are only minimally voltage dependent, and are closed by heptanol and high CO<sub>2</sub> exposure [6]. They also are capable of forming heterotypic channels with Cx40 and Cx43 that show asymmetry in V<sub>j</sub>-gating; moreover, the heterotypic pairs show dramatically lower unitary conductances than Cx40 and Cx43 channels, consistent with compromised conductance due to pairing with a high resistance hemichannel [6].

### *Connexin37*

Connexin37 (Cx37) is an endothelial cell connexin [9] found throughout the vascular tree, including endocardial vessels, but is absent in other regions of the heart. During embryogenesis, Cx37 is more widely distributed and can be detected in the developing murine ventricular myocardium, as well as in the area of the conotruncal ridges and atrioventricular cushions [10]. Double knockout of Cx40 (also found in endothelial cells of the heart) and Cx37 show marked alteration in cardiac development, leading to septal defects. To date there have been no reports of biochemical or morphological defects in the Cx37 single knockout [11], suggesting that Cx40 in endothelial cells is capable of rescuing normal endothelial cell function in the Cx37 null mice. Channels formed by Cx37 display high unitary conductance (about 300 pS) and rather strong transjunctional voltage dependence [9]. They are blocked by intracellular acidification, heptanol, and other uncoupling agents [9, 12]. Cx37 channels have low anionic permeability [13].

### *Connexin40*

Connexin40 (Cx40) is found in multiple areas of the heart including the endothelial cells and the atrial myocytes. Connexin40 appears to be the second

most abundant gap junction protein in the heart and cardiovascular system (behind Cx43). In the heart, Cx40 is expressed in nodal tissue, in bundles within the conduction system and in the atrium, whereas in ventricular muscle it is almost completely absent except in the coronary vasculature [14]. In both muscular and elastic arteries, Cx40 expression is prominent in endothelial cells and may also be present in smooth muscle cells. In embryonic mouse heart, Cx40 is widely expressed at atrial and ventricular primordia at 11 days post-coitum; as embryonic development progresses, Cx40 expression is maintained in atrium, whereas its initial ventricular expression becomes confined to the differentiating conduction system [15, 16].

In Cx40-deficient mice, ECGs are abnormal, with evidence of conduction slowing and frequent atrial rhythm disturbance [17]. Most Cx40 null mice show disturbances in ventricular activation as well. Cx40 channels are modestly sensitive to transjunctional voltage, have unitary conductances of about 200 pS [18]. Their sensitivity to acidification is similar to Cx43 [12].

#### *Connexin45*

Until the discovery of Cx30.2, the specialized conduction system of the heart was thought to contain only one isoform of connexin, connexin45 (Cx45). Early in development, Cx45 is found uniformly in the heart and decreases throughout development. In the adult mouse, localization of Cx45 is restricted to the atrioventricular node, the sinoatrial node, the ventricular conduction system, and in small amounts in the ventricular myocytes [19]. Some studies have indicated that there is an upregulation of Cx45 in ventricular myocytes in heart pathology, namely in heart failure [20]. The functional consequences of this upregulation may include a 'rescue' of loss of the main ventricular connexin, Cx43.

Cx45 channels are of low unitary conductance (30 pS), are cation selective, and exhibit strong transjunctional voltage dependence [21]. Sensitivity to acidification is higher than for most other connexins [12].

#### *Connexin43*

The most abundantly expressed connexin in the heart is Cx43. It is almost ubiquitously expressed, including in the ventricular conduction system in humans although only in the distal conduction systems in mice and rats [22]. The primary location of Cx43 is between the ventricular myocytes in normal heart, with a loss of Cx43 being a consequence of most types of heart pathology. In normal ventricle, Cx43 is primarily localized to the intercalated disks between myocytes. Some Cx43 immunolabeling is found to be present in the perinuclear region, co-localizing with endoplasmic reticular structures. This pool of Cx43 is presumably in the process of being oligomerized and transported to

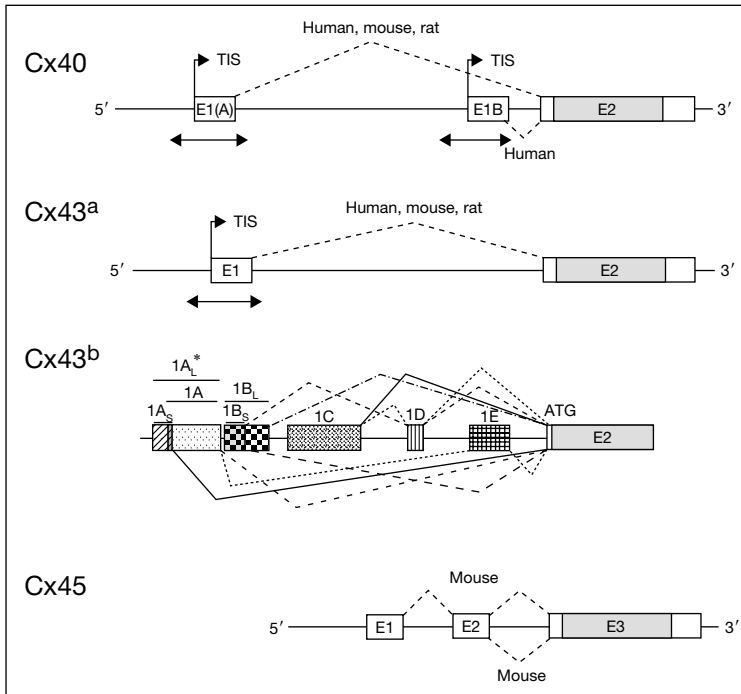
the cell membrane. Under ischemic conditions Cx43 shows an early dysregulation in localization [23] and in long-term cardiac disease there is altered Cx43 expression and distribution [24]. In the canine model, early as 3 h post-infarction there is a strong lateralization of Cx43 and an increase in immunolabeling on the lateral myocyte membranes [unpubl. obs.]. This change lasts for at least several days. One question has been whether this lateralized connexin protein is indicative of functional gap junctions, or if it is clusters of 'closed' connexin channels or hemichannels localized on lateral membranes. Using electron microscopy, we see an increase in small gap junctional plaques in the lateral membranes of ischemic myocytes at 5 days post-infarct [unpubl. obs.], suggesting that there is an increase in coupling in the side-to-side direction, but direct measurement of coupling in the side-to-side direction of cells from the surviving epicardial border zone of infarcted ventricle indicates that coupling is decreased in this direction rather than increased [25]. Overall, loss of coupling would be expected to increase anisotropy and provide a substrate for arrhythmogenesis. In contrast to the ventricle, Cx43 is upregulated in a number of pathologies of the atrium [26], lateralization of Cx43 expression in atrium was noted in patients with atrial fibrillation and was induced in rat atrium by rapid pacing for 24 h [27]. Whether this is a consequence or an underlying cause of fibrillation is still a matter discussion.

Cx43 null mice die at birth due to right ventricular outflow tract obstruction, resulting from impaired migration of neural crest derivatives during embryogenesis [28]. Ventricular activation patterns are impaired in late embryogenesis and neonatal homozygotes exhibit arrhythmogenesis [29].

Cx43 gap junction channels formed from Cx43 exhibit low to moderate voltage dependence unitary conductance of about 100 pS and are quite permeant to anions. They exhibit moderate pH sensitivity [12] and unitary conductance is altered by channel phosphorylation [30]. Channel function is also altered by protein-protein interactions, particularly by a variety of kinases (see below).

## **Transcription of Connexins and Gene Structure and Regulation**

The precisely localized regional expression of connexins in heart, the well-orchestrated developmental sequence of their expression, and selective changes in expression of individual connexins in response to pathophysiological stimuli all imply that the genes encoding cardiac connexins contain multiple tissue-specific and signal transduction responsive promoters. Teunissen and Bierhuizen [22] have recently reviewed the literature regarding regulation of the major cardiac gap junction proteins, Cx40, Cx43 and Cx45, and have summarized gene structures and what is currently known regarding transcription factor binding



**Fig. 2.** Gene structure and splicing methods of Cx40, Cx43 and Cx45. Shaded regions represent coding regions of the connexins, arrows indicate transcription start site and species information. Note multiple splicing sites for Cx40 and Cx45 and complexity of Cx43 splicing (Cx43<sup>b</sup>), in contrast to previous view of a single mRNA (Cx40, Cx43<sup>a</sup>, and Cx45 from [22], Cx43<sup>b</sup> [32]).

sites, as shown in figure 2. Briefly, each of these genes possesses two (Cx43; but see 30) or three exons (Cx40 and Cx45), with the most distal exon containing the entire coding sequence. For Cx40, the major transcript appears to be that initiated by the most proximal exon, although in humans a shorter transcript initiated at E1b may also be present [31]. For Cx45, there appear to be multiple transcripts, depending on which exon is used for transcription initiation and whether exons are spliced out in the process; interestingly, the longest transcript (initiated at the first exon) appears to be the least abundant [32]. For Cx43, originally reported to have a single intron [22], Pfeifer et al. [33] have recently reported a more complex structure with a total of five exons, generating nine distinct mRNAs. Evaluation of strength of actions of the individual promoter regions upstream of each transcription start site indicated that the promoter upstream of the first exon is the strongest, although it should be noted that this

strength is exceeded by the sum of activities of all other promoters [33]. As indicated below, the three active promoters contain different subsets of putative transcription binding sites, allowing the possibility that transcript abundance for different Cx43 mRNAs may be differentially regulated.

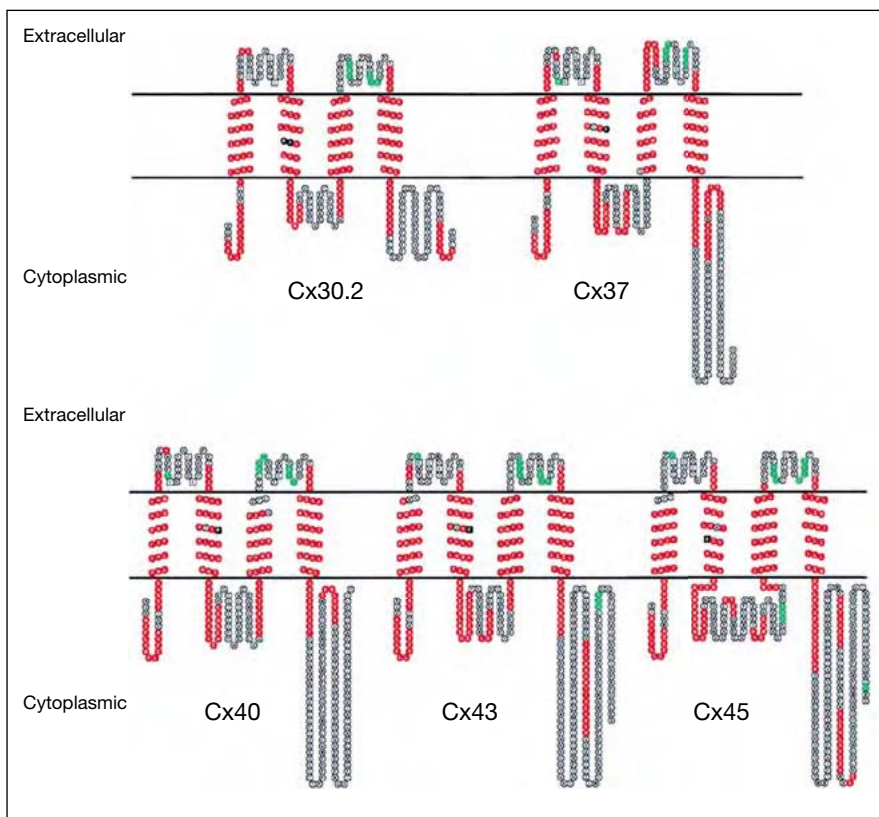
Promoter regions have been mapped for Cx40, Cx43 and Cx45 [22] (fig. 2). For Cx40, positive regulation upstream of the transcription start site has been demonstrated, and strong negative regulation by the downstream region [34] in rat, five AP1/SP3 binding sites have been identified for positive regulation [35]. The human Cx40 gene possesses highly homologous promoter regions, although an additional promoter close to an alternative transcription start site has been determined to be active in certain cell types [31]. It has been suggested that cell type specificity of Cx40 may be determined in part by the transcription factors Tbx5, Nkx2.5 and HF-1b [for overviews, see ref. 22, 36].

For Cx43, the promoter consists of a region of about 150 nucleotides spanning the first transcription start site, which contains Sp and AP1 binding elements, with Nkx2.5 having strong regulatory activity [22]. As indicated in the Teunissen and Bierhuizen review, a large number of signals have been demonstrated to regulate Cx43 promoter activity, either through individual response elements (e.g. estrogen, prostaglandin E<sub>2</sub>, cAMP, TCF/LEF, thyroid hormone) or through action on AP1-binding sites (e.g. protein kinase C through c-jun and c-fos). Because c-jun terminal kinase, a member of the mitogen-activated protein (MAP) kinase pathway, is activated under ischemic conditions and in overload hypertrophy, this suggests a mechanism for Cx43 downregulation under these conditions [37]. As indicated above, the presence of different sets of transcription factor binding sites in the alternative Cx45 promoters offers the possibility for alternative control of different mRNAs under different conditions; consequences for cells expressing different Cx43 RNAs remain to be carefully determined [33].

#### *Are Connexins also Transcription Factors?*

The appearance of a carboxyl terminal Cx43 fragment in the nucleus [38], the inhibition of DNA synthesis by Cx43 [37], the suggestion that Cx43 may be involved in gap-junction-independent processes (so-called ‘functions without junctions’), and recent studies showing profound changes in the transcriptome of Cx43 null hearts [40], all provide indirect evidence that Cx43 may influence expression of other genes. In the case of the Cx43 null heart, affected gene expression extends to all functional categories of encoded genes; moreover, the coordination of Cx43 expression with other genes in wild-type hearts accurately predicted many of the genes downregulated in the Cx43 null heart, indicating that expression of these genes is linked in normal hearts [40].





**Fig. 3.** PORTER sequences for each of the cardiac connexins. Red circles denote regions within an  $\alpha$ -helix, green circles indicate regions of  $\beta$ -sheet, and black circles are conserved prolines. In addition, extracellular cysteines not within  $\beta$ -sheets that are thought to be involved in interactions between connexin molecules across the extracellular space [76] are denoted by white boxes.

### Structure of Individual Connexins

Overall, individual connexin isoforms have high structural homology. They are all four-transmembrane-domain proteins with an intracellularly localized amino and carboxyl terminus (CT) (fig. 1). The cardiac connexin structures (based on Porter predictions [41], are shown in figure 3 and show high correlation with structures obtained by nuclear magnetic resonance (NMR) [42–45] and circular dichroism [Fort and Spray, unpubl.], including  $\alpha$ -helices within the amino terminus [46] and  $\beta$ -sheets within the extracellular loops. It is

interesting to note that the predicted locations of  $\alpha$ -helical and  $\beta$ -sheet structures for the three connexins suggest that there are common structured regions of these connexins, suggesting that these structures may be necessary for channel gating or folding regardless of dissimilarities in amino acid sequences.

Studies from our lab in collaboration with Paul Sorgen and Mario Delmar have examined detailed NMR signatures from the cytoplasmic loop (CL) and CT of Cx43 and demonstrated that at low pH, the CL structure contains significant  $\alpha$ -helical structures [42]. The Cx43 CL studied (residues 119–144) presents two  $\alpha$ -helices on residues 122–129 (NVEMHLKQ) and 136–143 (KYGIEEHG) [42]. In contrast, the portion of CT of Cx43 studied (residues 244–382) consists of two strong  $\alpha$ -helical domains within segments corresponding to 315–326 (AEQNR-MGQAGST) and 340–348 (DNQNSKKLA) [43; BMRB accession No. 5313], regardless of pH.

Structural changes within connexins may also be regulatory. We have shown that binding of the SH3 domain of c-Src (residues 274–283 of Cx43) [47–49] decreases the affinity of Cx43 for its scaffolding protein zonula occludens-1 (ZO-1) [50]. We have hypothesized that this occurs by alteration of the Cx43CT structure downstream of the SH3 binding site [44]. Recent studies indicate that regions of the Cx43CT are involved in dimerization of Cx43 proteins (aa regions 281–295, 299–304, 314–327 and 342–348 [45]). The function of this dimerization is not yet clear.

Using CD and protein prediction software we have mapped the entire cytoplasmic and extracellular domains of Cx43 [Fort and Spray, unpubl.]. The  $\alpha$ -helical assignments we obtained for the CL and CT at pH 7.4 in a solution containing 30% 2,2,2-trifluoroethanol (TFE, an agent that stabilizes secondary structure) were similar to the NMR values. Other  $\alpha$ -helical regions found were on the amino terminus and the two extracellular loops (EL), closely matching the predictions. We additionally observed  $\beta$ -sheet structures on the two ELs and on the CT of Cx43. The regions most divergent between these connexins are the CL and the CT. Of the two regions with structure in Cx43, only the first  $\alpha$ -helix in the CT is similar to what is seen in Cx40. However, in both connexins there is an  $\alpha$ -helix predicted at the beginning of CL and the CT. The CT proximal region has not been studied in Cx43, but the first region of the CL shows strong  $\alpha$ -helical content with CD under conditions where water molecules are stripped from the peptide (100% TFE).

The highest resolution structure currently available for a connexin isoform is for the transmembrane domains of Cx43. In these studies, 2-dimensional crystal cryo-electron microscopy of CT-truncated Cx43 (Cx43: amino acid residues 1–263) was used to resolve the transmembrane domain regions to 0.6 nm [51–54]. Together with studies summarized above, the Cx43 molecule appears to have four  $\alpha$ -helical transmembrane domains, EL that are largely  $\beta$ -sheet with possibly minor

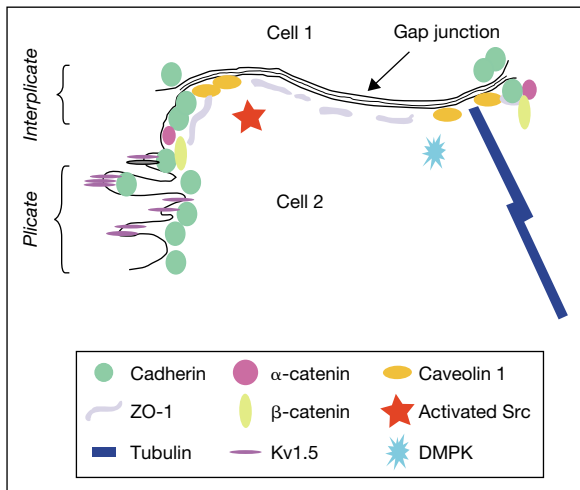
$\alpha$ -helical content, and small helical segments in the amino terminus and CT and, under acidifying conditions, an  $\alpha$ -helical segment in the CL region.

### **Protein Interactions of Connexins**

Traditionally, connexin proteins were thought to bind only to themselves and to other connexin proteins (providing both homotypic and heterotypic interactions between different connexin isoforms). This view was abruptly altered by the discovery of interactions of one connexin isoform (Cx43) with the scaffolding protein ZO-1 [55, 56]. From these initial descriptions of protein-protein interactions of connexins there has been an explosion of studies examining binding partners for connexins [57, 58]. The roles of these binding partners are not yet clear but some studies indicate that protein-protein interactions of connexins are important in the regulation of gap junction size and function [50, 59]. To date, only two of the cardiac connexins have been found to have binding partners, Cx43 and Cx45. Cx45 has been shown to interact with ZO-1, but no other binding partners have been found. In contrast, Cx43 is known to have multiple binding partners that may aid in the formation and regulation of the gap junction channels (fig. 4).

In the intercalated disk, there are three major junctional types in close proximity, including desmosomes and adherens junctions as well as gap junctions. While interaction of Cx43 with cadherins is now well known [60, 61], the localization of desmosomes in close contact with both adherens junctions and gap junctions suggests that further studies may find a link between all three junctional types. Formation of such a junctional triad would presumably strengthen the entire intercalated disk complex, thereby providing a structure that is less likely to break down except in cases of severe pathology. A major component of the intercalated disk known to interact with Cx43 is N-cadherin. Initial studies showed that the presence of cadherin was required to bring cells into close contact with each other, allowing gap junctions to form between the cells [62, 63]. Later studies both confirmed the requirement for cadherins in the formation of the intercalated disk structure [64], and as an interacting partner with Cx43 [60, 61]. Whether this is a direct interaction or whether they form a complex with other proteins is unclear, although since they do not directly overlap at the intercalated disk it may be that the interaction is indirect. If these proteins interact only via a complex, it is likely that the interaction is mediated through the scaffolding protein ZO-1 which binds to both N-cadherin and Cx43.

The first protein-protein interaction of a cardiac connexin found was between Cx43 and the scaffolding protein ZO-1. ZO-1 is a member of the MAGUK family of scaffolding proteins. Cx43 interacts via the last 11 amino



**Fig. 4.** The Cx43 nexus at the intercalated disk. Cartoon shows an intercalated disk consisting of a plicate or step-like segment containing membrane projections, adherens junctions and desmosomes, and an interplicate segment with an associated gap junction (delineated by the two black lines). Proteins associated with different segments of the intercalated disk are indicated. Note that the nexus of the gap junction protein and its binding partners are a large complex that includes interaction with protein partners of cadherin, the main protein component of adherens junctions.

acids of its CT domain to the second PDZ domain of ZO-1 [45]. Under normal conditions, the binding of ZO-1 to Cx43 is a high affinity interaction, with  $K_d$  in the range of 400 nM [50]. This affinity can be altered greatly under pathological conditions such as acidification (see below). The importance of the Cx43/ZO-1 interaction is unclear, but studies from the Gourdie lab have shown convincingly that at least one function of the Cx43/ZO-1 interaction is to control the size of Cx43-containing gap junctional plaques [65]. This suggests that under pathological conditions when the Cx43/ZO-1 interaction is altered, the gap junctional plaque morphology may change, as well as gap junction function.

A number of other proteins are now known to interact with Cx43, including the tyrosine kinase c-Src, the cytoskeletal protein tubulin, signaling proteins p120 catenin and  $\alpha$ -catenin, caveolin-1 which is a component of lipid rafts, other ion channels such as the potassium channel Kv1.5, and myotonic dystrophy protein kinase (DMPK) [60, 61, 66–70]. All of these proteins are found in cardiac myocytes, localized to the intercalated disk, and they presumably interact with Cx43 under various intracellular conditions. Their role in Cx43 regulation is unclear, although some studies have shed light on possible functions for these protein-protein interactions. The tyrosine kinase c-Src has been known for

a long time to interact with Cx43 and regulate the activity of the channel [66, 70]. Either activation of c-Src or introduction of viral-Src (v-Src), a constitutively active form of c-Src can cause closure of Cx43 channels [70]. c-Src is activated under a number of pathological conditions including intracellular acidification [71], an event known to occur in myocardial ischemia. Upon activation, the SH3 domain of c-Src binds to the proline-rich region of Cx43 and causes a shift in the structure of the Cx43CT [45], leading to a decrease in the affinity of the Cx43CT for the PDZ domain of ZO-1. Thus, activation of c-Src regulates the Cx43/ZO-1 interaction, causing Cx43 to unhook from its scaffold [50]. We hypothesize that the unhooking of Cx43 from ZO-1 allows for Cx43 to move away from the intercalated disk under pathological conditions and become more randomly localized around the membranes of the myocytes.

The interaction of tubulin with Cx43 is presumed to have less of a regulatory function and more of a trafficking function. Tubulin is thought to interact with Cx43 on the CT domain near the emergence of this domain from the plasma membrane (aa 228–263) [67]. The tight interaction of tubulin with Cx43 is suggested to aid in the localization of Cx43 at the intercalated disk rather than at the lateral membranes of myocytes. Many pathological conditions cause lateralization of Cx43 in myocytes, including myocardial infarction and cardiac hypertrophy [72, 73], suggesting that these conditions may cause a dysregulation of the Cx43/tubulin interaction. Further studies should include examination of how the Cx43/tubulin interaction changes during alterations in cardiac function.

Members of the catenin family of proteins, both  $\alpha$ - and  $\beta$ -catenin, have been shown to interact with Cx43. These proteins also interact with cadherins, and are important in the function of the adherens junctions.  $\alpha$ -Catenin and its interaction to N-cadherin are important for formation of the intercalated disk in myocytes; and  $\alpha$ -catenin does not colocalize with Cx43 until disks are formed [74]. While  $\alpha$ -catenin has signaling functions when interacting with cadherins, the function of the interaction between Cx43 and  $\alpha$ -catenin is unclear. Formation of the complex of Cx43 and  $\beta$ -catenin, on the other hand, has been shown to increase the transcription and translation of Cx43 producing functional channels at the plasma membranes and increasing cell-cell coupling [60].

There is limited information of the interaction of Cx43 with caveolin-1, Kv1.5, and DMPK, but studies indicate the possibility that these intercalated disk proteins also interact, possibly directly, with Cx43 [68, 69, 70]. This wide variety of binding partners suggests that the complex associated with Cx43 must be quite large. Interestingly, when Cx43 immunoprecipitates from heart tissue are run on gels and subjected to silver staining for detection of all proteins, multiple protein bands are seen ranging from 15 to 200 kDa [unpubl. obs.]. Almost 20 years ago, electron microscopy studies by Ernest Page's laboratory [75] described a 'cytoplasmic surface fuzz' on isolated ventricular gap

junctions that could correspond to this ‘nexus’. These studies may thus have foreseen the now well established concept that connexins, like many junctional proteins, function through interactions with protein partners.

## Perspectives

Gap junctions in the heart provide rapid impulse propagation along the conduction system and coordinate rhythmic contraction of the muscular chambers. Changes in abundance and distribution of the cardiac gap junction proteins occur as a consequence of cardiac disease and these changes are likely causes of resulting arrhythmias. Frontiers of understanding (and ultimately reversing) these alterations in connexin expression and distribution include the development of pharmacological tools to manipulate gap junction channel opening, closing and response to stimuli, determination of components of the nexus of connexins and interacting proteins and how this complex changes in different cellular compartments, atomic level structural information on membrane-spanning extracellular and intracellular connexin domains and changes upon channel formation, gating and inter- and intramolecular binding. In addition, the accumulation of detailed knowledge of gene organization of the cardiac connexins should provide understanding of how cardiac genes are turned on and off (and more subtly modulated) with developmental and cell type specificity and under pathological conditions. Further high throughput analysis of normal, connexin-altered and cardiomyopathic cardiac tissue should determine the extent to which gap junction gene expression drives or inhibits expression of downstream gene profiles that may be targeted for reversal by altering expression of key genes in the pathway.

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## Physiology of Cardiovascular Gap Junctions

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### Abstract

Cardiac gap junction channels are crucial for conduction of the electric impulse. Between cardiomyocytes there exist gap junctions constructed from connexin40 (Cx40), Cx43 and Cx45. A fourth isoform, Cx37, is expressed in the endothelial lining. Each of these channel types possesses specific properties and their functioning is regulated by various mechanisms. In this chapter we compare the physiological differences between these channels and discuss the factors involved in modulation of channel properties. Next, we evaluate how alterations in expression and differential regulation of channel properties affect cardiac impulse propagation.

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Every heartbeat triggered by spontaneous depolarization of myocytes within the sinoatrial node results from a highly coordinated interplay of molecular and cellular events. In order to contract in synchrony, the electrical impulse must be propagated rapidly from cell to cell all over the subsequent cardiac compartments. Impulse propagation is mediated by current flow through clusters of intercellular channels called gap junctions. Each gap junction channel is constructed from two half-channels (connexons) aligned head to head, contributed by both participating cells. Each connexon is made of 6 hexagonally arranged proteins called connexins (Cx) which form a large family of highly related transmembrane proteins. At least three members are expressed in mammalian cardiomyocytes: Cx40, Cx43 and Cx45. In most species, atrial myocytes express Cx40 and Cx43, ventricular myocytes predominantly express Cx43 while Cx45 is expressed at very low levels both in the atria and ventricles. All three isoforms can be found in myocytes composing the specialized conduction system and in vascular smooth muscle cells. Cx37, a fourth cardiac isoform, is

co-expressed with Cx40 and Cx43 in the endothelial lining of the vessels. Although low expression levels of other isoforms in the heart have been claimed, they will not be further discussed in this chapter.

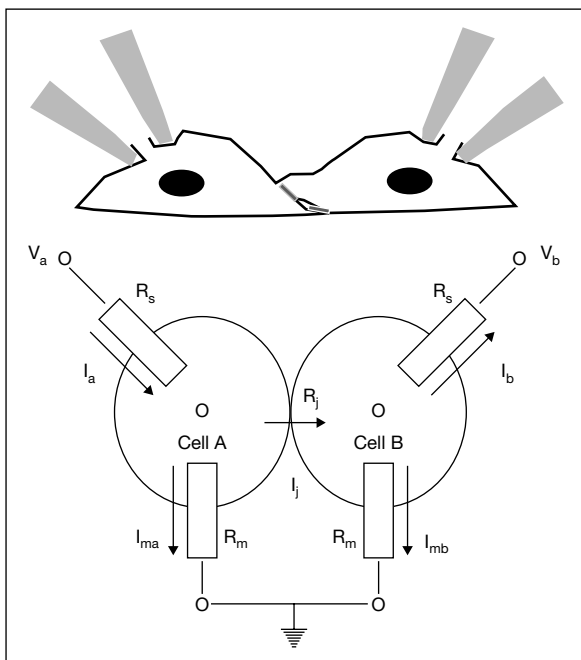
The electrical and metabolic coupling of cells is determined by the number of channels expressed, the open probability of these channels and the conductance (or permeability) of each single channel. These characteristics highly depend on the connexin isoform involved. Channels composed of different connexins possess different properties which are, in addition, susceptible to differential regulation. The structural background for this are differences between the connexin isoforms in amino acid sequence, especially within the intracellular domains of the protein. Here we find amino acid residues involved in pH and voltage sensitivity and consensus sequences susceptible to phosphorylation by different classes of protein kinases.

Expression of more than one connexin isoform within a cell might give rise to heterotypic/heteromeric channel formation. The ability of different isoforms to integrate into functional channels is mainly determined by the degree of homology of the amino acid sequence and structure of the second extracellular loop. Homotypic channels can consist of two identical and symmetrical connexons (either homomeric or heteromeric connexons) with regard to the plane of separation of the gap junction.

Heterotypic channels consist of two dissimilar connexons (either homomeric or heteromeric as well). This organization theoretically allows a plethora of different channel types with a high level of complexity. To study the physiology of channels composed of different isoforms, genes encoding the cardiac connexins have been characterized, cloned and introduced in noncommunicating tumor cells or *Xenopus* oocytes. Such reduced cell systems overexpressing (exogenous) gap junction channels composed of one or more types of connexins allow for a controlled evaluation of single channel properties and some of the modulating factors affecting these properties. In this chapter, we will summarize information on the physiology and modulation of 'cardiac gap junctions' as obtained from experiments using transfected cells. In addition, these observations are compared with data obtained from isolated cardiomyocytes and extrapolated to impulse propagation through the heart.

## Methods and Results

Intercellular coupling concerns macroscopic (electrical) conductance ( $g_j$ ) and metabolic coupling. Both parameters are determined by the number of expressed channels ( $N$ ), single channel conductance ( $\gamma_j$ ) or permeability, and the open probability of a single channel ( $P_o$ ). Single channel properties can be



**Fig. 1.** Upper panel: schematic representation of dual whole cell recording. Two pipettes are sealed to the membranes of a cell pair, the patches under the membranes are broken, giving access to the cell interiors. The cells are electrically coupled by means of gap junctions. Lower panel: equivalent resistive circuit of dual whole cell recording.  $R_s$  = Access resistance to the cell;  $R_m$  = membrane resistance;  $R_j$  = gap junctional resistance;  $V_a$ ,  $V_b$  = command potentials of voltage clamp amplifiers;  $I_a$ ,  $I_b$  = holding currents of both voltage clamp amplifiers;  $I_{ma}$ ,  $I_{mb}$  = membrane currents;  $I_j$  = gap junctional current.

affected by modulation of  $\gamma_j$  but also by changes in  $P_o$  of the channels. Generally, gap junction channels are freely permeable for molecules up to a size of approximately 1 kDa. This includes movement of ions, involved in transduction of electrical signals, but also of small metabolites, nucleotides and second messengers. Metabolic coupling has a critical function in embryo- and organogenesis, differentiation and cell homeostasis.

Electrical coupling of cells can be studied using the dual voltage clamp technique [1]. The basic principle of this technique is schematized in figure 1. Patch electrodes are placed on the membrane of each cell of a cell pair. By forming a 'giga-seal' between electrode and cell membrane and breaking the membrane patch under the pipette tip, an electrical circuit is obtained in which one cell can be 'clamped' at a fixed membrane potential while the membrane potential in the other cell can be changed. This generates a voltage difference between

the cells. In this way one can control voltage and measure current simultaneously, allowing calculation of the junctional conductance ( $g_j$ ) from the trans-junctional voltage difference applied and the junctional current measured in the cell clamped at a fixed membrane potential. Instead of breaking the membrane under the pipette tip, one can add pore-forming antibiotics (e.g. amphotericin B) to the pipette solution. In this approach, named perforated patch, these agents initiate the electrical circuit by generating small pores in the membrane thereby preserving a more physiological composition of the intracellular fluid. Infusion into the bath solution of agents as heptanol or halothane leads to uncoupling of gap junctions by dramatically reducing the gating of individual channels to a level at which single channel gating can be observed. By this, single channel conductance ( $\gamma_j$ ) can be calculated. This approach has been used worldwide to investigate the physiological characteristics of gap junction channels.

### *Electrical Properties*

Obviously, the conductance of a single ionic channel, and thus a gap junction channel, is dependent on the composition of the internal pipette-filling solution [2]. Generally, potassium gluconate as charge carrier reveals a lower unitary conductance as compared to potassium chloride or cesium chloride. This can be explained by the higher conductance of the small  $\text{Cl}^-$  anions compared to the rather large gluconate anion. Therefore, in cells transfected with e.g. Cx40, a variety of large conductance states of 120–200 pS and less frequently, of approximately 30–40 pS has been reported [3–6]. These channels appear mildly sensitive to the transjunctional voltage gradient ( $V_j$ ) with a half-maximal inactivation at  $\pm 50$  mV [4, 7]. In spite of the large homology of Cx40 in species as the rat, mouse and human, pronounced differences in properties can be found. Rat Cx40 (rCx40) transfected in N2A cells appeared to be cation selective while this was not found for mouse Cx40 (mCx40) [4, 8].

Compared to Cx40 channels, Cx43 channels are even less sensitive to  $V_j$ . Conductance starts to decrease when  $\Delta V_j$  exceeds  $\pm 40$  mV, and half-maximal inactivation occurs at  $\pm 60$  mV [9]. On the other hand, unlike Cx40 channels, Cx43 channels are not ion selective [8]. rCx43 and human Cx43 (hCx43) channels exhibit three  $\gamma_j$ s; a major state at 40–60 pS, and two states of minor occurrence of 20–30 and 70–100 pS with K-gluconate in the pipette. Remarkably, in mCx43 only the two largest states have been detected [3, 10, 11]. In pairs of neonatal cardiomyocytes, junctional coupling is variable in size which is partly dependent on the culture time. Unitary conductances show a main  $\gamma_j$  of 40–45 pS, but a substate of 20 pS is found as well [12, 13]. Dephosphorylation of these channels increases  $\gamma_j$  to 70 pS [13]. Although channels of other connexins might

be present (knowing that rat neonatal cardiomyocytes can express Cx40, Cx43 and Cx45), the measured  $\gamma_j$ s closely resemble those described for Cx43 channels in transfected cells. In freshly isolated adult myocytes, guinea pig and rabbit ventricular cells express channels insensitive to  $V_j$  with a main  $\gamma_j$  of  $\sim 100$  pS (using CsCl as charge carrier), again presumably reflecting Cx43 channels [14, 15]. In rabbit atrial cells, besides the 100 pS conductance a larger conductance of 185 pS was measured which likely represents the gating of Cx40 channels [15].

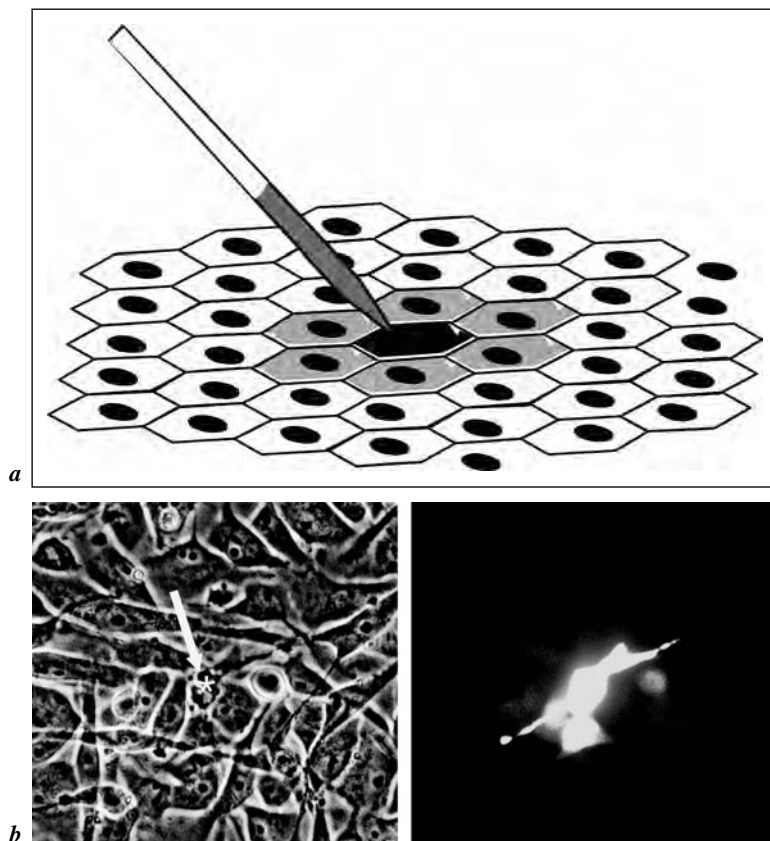
Cx45 is the third isoform expressed in cardiomyocytes. As compared to Cx40 and Cx43, Cx45 channels are steeply voltage dependent with half-maximal activation at  $+/-20$  mV [5, 16]. Chicken Cx45 channels appeared highly selective for cations [17]. Unitary conductances are small, with values of about 20 and 40 pS [11, 17, 18].

Cx37 is not expressed in cardiomyocytes but forms functional channels between endothelial cells. Expressed in N2A cells, human Cx37 channels appeared moderately voltage sensitive with a half-maximal inactivation at a  $V_j$  of  $+/-28$  mV [19]. The channels have a low anion permeability [8], and a rather large unitary conductance of about 300 pS with a minor substate of 63 pS [20]. Point mutations (V156D and K162E) in the pore lining segment M3 result in a variety of conductive states generally smaller than the native one. These mutations also decrease voltage sensitivity [21].

As expected based on sequence homology, at least in transfected cells, heterotypic channels have been shown to exist functionally. Being the most abundantly expressed isoform in multiple tissues and cells, Cx43, seems most capable of forming heterotypic channels with several isoforms including Cx37, Cx40 and Cx45 [22]. Early reports already described functional channels for Cx37/Cx40, Cx37/Cx43, Cx40/Cx45 and Cx43/Cx45 [7, 23, 24]. Two other studies suggested the formation of both heterotypic and heteromeric Cx40/Cx43 channels [25, 26], which was in contrast with earlier observations [7, 27, 28]. Biochemically, both Cx43/Cx45 and Cx40/Cx43 can be co-immunoprecipitated, suggesting a direct connection [29]. As expected, heterotypic and heteromeric channel properties differ from those of the two participating connexins when expressed as a homotypic channel [22, 25, 26, 30], thereby indeed creating a plethora of different channel characteristics [reviewed in ref. 31]. Whether these different heterotypic channels are actually present in vivo remains to be elucidated.

### *Metabolic Coupling*

Metabolic coupling is commonly evaluated by the passage of small fluorescent (dye) molecules [32]. To assess the size limits of metabolites capable of moving from one cell to another is not that simple using this approach. Diffusion



**Fig. 2.** *a* Illustrative representation of dye injection into one cell within a monolayer of cultured cells using a sharp microelectrode. Dye transfer through gap junctions to the surrounding cells is used to assess metabolic coupling. *b* Dye permeability of LY in a culture of SkHep1 cells expressing human Cx40. Left panel: phase contrast view of the cells with indicated (asterisk and arrow) the injected cell. Right panel: transfer of LY to the neighboring cells after 5 min. The number of stained cells can be counted by combining the phase contrast view and the view of diffused dye.

characteristics not only depend on the size of the tracer used, but also on the charge of the molecule. Most commonly used tracers are Lucifer Yellow (LY, 443 Da), 2'7'-dichlorofluorescein (DCF, 401 Da) and 6-carboxyfluorescein (6CF, 376 Da). The fluorescent dye (dissolved in the internal pipette solution) is injected into one cell of a monolayer of cultured cells with a sharp microelectrode (fig. 2a). Transfer of dye through gap junctions is assessed after a fixed



period of time by counting the number of fluorescent cells. Figure 2b (right panel) shows the spreading of LY to neighboring cells from the injected Cx40 expressing cell in the left panel (marked with an asterisk). Based on this principle, the scrape-loading technique has been used as well. With this approach, a demarcation line of ruptured cells is created in a monolayer of cells cultured under low calcium conditions in order to avoid closing of the channels between the ruptured cell and the neighboring cells. The fluorescent dye added to the culture medium diffuses into the ruptured cells. Transfer to the neighboring cells serves as a measure of coupling.

Small molecules like propidium iodide or ethidium bromide easily pass nearly all connexin channels including those built of Cx37, Cx40, Cx43 and Cx45 [23]. Dye permeability of Cx43 channels seems most successful and has been shown for LY, DCF and 6CF [3, 8, 23, 33, 34]. As with voltage sensitivity, interspecies differences exist also with respect to dye transfer. Veenstra et al. [8] showed pronounced differences between rCx43 and chicken Cx43 in ability to pass 6CF. Studies on the passage of dyes through channels composed of Cx40 or Cx45 are less complete. Both hCx40 and mCx40 channels are permeable to LY [6, 23], while rCx40 is permeable to 6CF and displays limited permeability to 6CF [4, 8]. mCx45 but not hCx45 channels appear moderately permeable to LY [11, 23, 34]. Veenstra et al. [17] showed that hCx45 channels are also permeable to DCF but not to 6CF, which can be explained by the difference in charge of the molecules. Dye transfer follows a far from linear relation with the size of single channel conductance and thus does not seem to be just a matter of ‘diffusion through an aqueous pore’.

Cx37 channels which have a rather large unitary conductance only transfer dyes with a restricted size and with much lower efficiency as compared with, e.g., Cx43 channels [35]. Cx40 and Cx45 channels behave in an intermediate fashion while in heterotypic Cx43/Cx37 channels, the constituent with the most restricted dye transfer (Cx37, based on homotypic channels) determined the capability of dye transfer [36].

## *Modulation of Channel Properties*

### *A: Voltage and Chemical Gating*

The specific characteristics of the gap junction channels differ between isoforms but also, for several isoforms, between species. Moreover, these characteristics are sensitive to modulation by several factors as transmembrane voltage gradient, internal pH/pCa and phosphorylation state of the constituting connexin molecules. As we shall see, especially the carboxy terminus of the

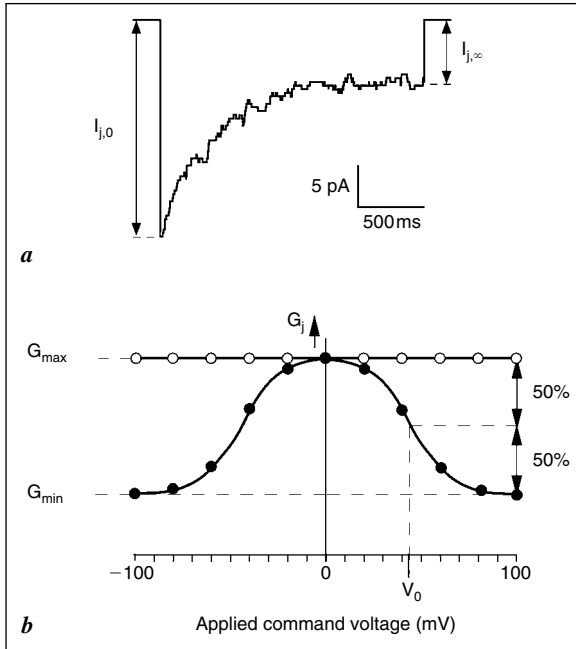
connexin molecules appears to be of crucial importance in several modes of regulation.

Intracellular calcium level has been recognized long ago to be an important modulator of gap junction gating [37]. Obviously, when cells are ruptured and no longer able to maintain calcium homeostasis, closure of gap junctions prevents deterioration of neighboring cells and tissue. The exact intercellular calcium concentration required for complete closure still remains a subject of discussion and seems to vary between different cell types. It is unlikely that clustered calcium ions directly induce gating since other positively charged ions were unable in this. They more likely act through a soluble intermediate for which calmodulin is an important candidate [38, 39].

Protonation of three conserved histidine residues in the L2 region (amino acids 119–144) of the cytoplasmic loop might be required in the process of pH gating [40–43]. In Cx43 channels, these histidines may serve as a receptor in the pore for yet unidentified parts of the carboxy terminus in order to facilitate pH gating according to a ‘ball-and-chain’ model [44, 45]. Dimerization of carboxy-terminal domains within the hemichannel is possibly involved in closing of the pore upon lowering of pH [46]. The ball-and-chain model was also postulated for Cx37 and Cx40 but not for Cx45 as truncation of the carboxy terminus in Cx43 channels makes them insensitive to acidification whereas Cx45 channels with a truncated carboxy terminus remain sensitive to low pH gating [44, 47]. A cooperative interaction has been described for Cx40 and Cx43 which increases the pH sensitivity of those channels [48].

Within the physiologically relevant range of intracellular pCa and pH it is unlikely that  $\text{Ca}^{2+}$  or  $\text{H}^+$  independently strongly affect the conductive properties of Cx43 channels [42, 49]. However, in some cells there is evidence that gating behavior is affected by intracellular pH and pCa at near physiological concentrations, indicative of a regulatory mode relevant to normal cell function [50]. Yet, a synergistic mechanism between  $\text{Ca}^{2+}$  and  $\text{H}^+$  that strengthens their effect on Cx43 channels could be of importance under conditions of cardiac ischemia when intracellular  $\text{Ca}^{2+}$  and  $\text{H}^+$  increase [51, 52]. Several studies show that especially under conditions of ischemia,  $g_j$  might be synergistically modulated by  $\text{H}^+$ ,  $\text{Ca}^{2+}$  and amphiphatic lipid metabolites [51–55]. The chemical gating of gap junctions is often referred to as a slow gating process which, as suggested by some investigators, is identical to the slow gating component seen in voltage-sensitive gating [56]. For a review on chemical gating of gap junction channels, see Peracchia [50].

The carboxy terminus, amino terminus, first transmembrane segment, first extracellular loop and a conserved proline residue in the second transmembrane segment, are reported to participate in the voltage-gating properties of the channels [57, 58]. Experimentally, when rather small transjunctional voltage steps



**Fig. 3.** *a* Current trace of the nonstepped cell of a computer-simulated cell pair, containing six Cx43 gap junction channels, as a result of a 100 mV command voltage step.  $I_{j,0}$  and  $I_{j,\infty}$  are the instantaneous and the quasi-steady-state junctional currents, respectively. *b* Diagram of instantaneous (open circles) and quasi-steady-state (closed circles) values of normalized conductance ( $G_j$ ) vs. the applied command voltages and a Boltzmann fit (solid line).

are applied to gap junctions, the conductance remains almost constant in time. Upon larger voltage steps, all cardiac gap junctions exhibit voltage sensitivity and decrease their conductance, which is seen as a relaxation of the junctional current, as shown in figure 3a. Simplified, the open probability within a population of channels decreases through fast but incomplete gating of individual channels, a process in which the carboxy terminus seems to be involved, possibly similar to pH gating, according to a ‘ball-and-chain’ model [59]. At the onset of a 100 mV command voltage step on a computer simulated cell pair, containing in this example six Cx43 gap junction channels, an instantaneous current  $I_{j,0}$  is seen in the nonstepped cell, which strongly diminishes during the 2-second step, reaching a quasi-steady-state value ( $I_{j,\infty}$ ) within 1–2 s. A common way to present and quantify voltage-dependent parameters of gap junctions is by plotting all the  $G_j$  values, normalized for the junctional conductance

measured at a small voltage step (e.g. 10 mV), for voltage steps from, e.g., -100 to 100 mV as presented in figure 3b. The data are fitted to a Boltzmann equation [60, 61].

$$G_j = (G_{\max} - G_{\min})/[1 + \exp(A \cdot (V_j - V_0))] + G_{\min}$$

This fitting procedure gives the value for  $G_{\max}$  and  $G_{\min}$  (the normalized maximal and residual junctional conductance, respectively),  $A$  ( $= n \cdot q/k \cdot T$ , where  $n$  is the equivalent number of electron charges  $q$  that move through the entire transjunctional field between open and closed states,  $k$  is Boltzmann's constant and  $T$  is the absolute temperature), and  $V_0$  (the value of half-maximal inactivation).

At a very large transjunctional voltage gradient, when theoretically all channels are in a 'closed' state, a limited level of conductance can still be detected. This residual conductance might be caused by the mentioned incomplete closure of the pore.

Supportive for the ball-and-chain model are studies showing that expression of Cx40 or Cx43 truncated from their carboxy terminus together with the separated Cx43-CT, restores the voltage gating sensitivity which was absent in cells solely expressing truncated channels [62, 63]. In addition, this implies that regions involved in this gating process are rather conserved.

Voltage gating can be influenced by the interaction of a connexon with its opposing connexon [64]. This mode of regulation becomes more complicated in gating behavior of heteromeric/heterotypic gap junction channels [for a review, see ref. 31].

### *B: Phosphorylation of the Connexin Protein Subunits*

The capability to modify channel behaviour through phosphorylation of the constituting connexins is largely determined by the amino acid sequence of the cytoplasmic loop (between transmembrane segment 2 and 3) and the C-terminus (CT). Here multiple amino acid motifs are found to be susceptible to phosphorylation by different intracellular protein kinases [65–68]. As an example, the different number of consensus sites for phosphorylation of Cx43 as expressed in mice, rats and humans are given; hCx43 contains 10 putative phosphorylation sites for PKC, 4 for PKA, 3 for PKG and 2 for MAP kinase. In rCx43 and mCx43, 14 sites for PKC, 3 for PKA, 4 for PKG and 3 for MAP kinase can be identified [65, 66]. The effect of phosphorylation of connexin molecules on modulation of channel properties can be studied in cultured cells using membrane permeable agents which directly activate the different protein kinases. Short-term application (up to 15 min) of these substances rapidly stimulates the involved protein kinases and because of the short application time excludes the possibility of alterations in expression. In addition, application of several extracellular (growth)

factors can be used to study receptor-mediated activation of kinases. Phosphorylation of connexins occurs predominantly on serine residues, but also on threonine and tyrosine residues.

#### B1: Phosphorylation of Cx43 Gap Junctions

Phosphorylation of Cx43 on serine residues, mediated by PKG, PKC, PKA and MAP kinase, enhances the prevalence of the smallest  $\gamma_j$ . In contrast, treatment of cells with agents promoting dephosphorylation, shifts  $\gamma_j$  to the largest state [10]. The permeability to dye molecules seems to follow these changes in  $\gamma_j$  although alterations in  $\gamma_j$  do not simply explain changes in  $g_j$  since phosphorylation might additionally affect  $P_o$ .

In most studies on phosphorylation of Cx43, the protein as shown on Western blot is separated into three bands with molecular weights ranging from 41 to 46 kDa. The lowest band represents the nonphosphorylated state (NP) while the other two slower migrating bands represent a phosphorylated state (P1) and a highly phosphorylated state (P2) [69]. Phosphorylation of Cx43 is not only involved in regulation of the channel properties but plays an important role in processes as trafficking, downregulation and insertion of the channels into the membrane [70]. Stimulation of PKC with the well-known phorbol ester TPA rapidly (hyper)phosphorylates Cx43 on Ser368 [71]. Prolongation of this stimulation approach results in internalization/downregulation of Cx43 and trafficking failure [72]. Similarly, phosphorylation of rCx43 on Ser255 accelerates internalization and degradation [73].

Stimulation of PKG (in rCx43 transfected cells) and PKC (in rCx43 and hCx43 transfected cells) reduces both dye permeability and  $\gamma_j$  [11, 74, 75]. Remarkably, PKG stimulation causes a reduction in  $g_j$ , whereas stimulation of PKC increases  $g_j$  although both decrease  $\gamma_j$ . This discrepancy can only be explained by a differential effect on  $P_o$  assuming that time limits a substantial change in the number of channels (as mentioned the effect of phosphorylation occurs within minutes while half-life of Cx43 is about 1–2 h [76]). Stimulation of PKG in cells transfected with hCx43 appeared ineffective. As compared with rCx43, the hCx43 coding region lacks one particular consensus site for modulation by PKG [74]. The substitution of Ala at position 257 in hCx43 instead of Ser in rCx43 explains why rCx43 is susceptible to phosphorylation by PKG and hCx43 is not.

Comparable to the effects observed in transfected cells, in rat cardiomyocytes acute stimulation of PKG reduces  $g_j$ ,  $\gamma_j$  and dye permeability [77, 78]. In line with the observations made in transfected cells, stimulation of PKC decreased  $\gamma_j$  and dye coupling but resulted in an overall increase in  $g_j$  [78]. However, regarding the effects induced by PKC stimulation in cardiomyocytes, other and conflicting observations have been described in the literature.

Similarly, using TPA to stimulate PKC, another report claims no effect [79], while a third one claims a decrease in  $g_j$  without changes in  $\gamma_j$  [80].

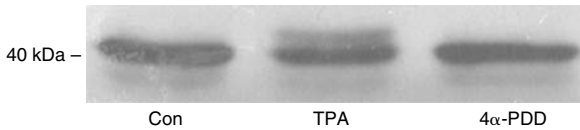
Experiments modulating the activity of the local renin-angiotensin system in cardiomyocytes in which angiotensin I (Ang-I) is converted by angiotensin-converting enzyme (ACE) to angiotensin II (Ang-II), are in favor of the last observation. Ang-II, the effective metabolite of the system, exerts its action through binding at AT-1 and AT-2 receptors, thereby activating PKC and MAP-kinases Erk1 and Erk2, respectively. De Mello [81–83] and De Mello and Altieri [84] have shown that administration of renin, Ang-I or Ang-II to adult rat myocytes rapidly reduced  $g_j$  due to activation of PKC [81–84]. In contrast, inhibition of ACE (thereby reducing the amount of Ang-II) increased  $g_j$  [83]. Phenylephrine binding to  $\alpha_1$ -adrenergic receptors also activates PKC and similarly to Ang-II decreased  $g_j$  [85].

Additionally, basic FGF (bFGF) reduced metabolic coupling as assessed by dye-transfer while phosphorylation of Cx43 was strongly increased mainly on serine residues but also on tyrosine residues [86]. On the other hand, anti-arrhythmic peptides tend to enhance gap junctional coupling [87, 88]. The expression of multiple PKC isoforms in cardiomyocytes is important to explain the differences in effects as induced by activation of PKC. As it appears, activation of different PKC isoforms may have different effects on junctional communication since stimulation of PKC $\alpha$  enhances junctional communication [87, 88], while stimulation of PKC $\epsilon$  reduces communication [89]. Phorbol esters as TPA activate both PKC $\alpha$  and PKC $\epsilon$ . A different PKC $\alpha$ /PKC $\epsilon$  activation ratio might explain the reported differences observed when myocytes are stimulated by TPA [90].

In transfected cells, activation of PKA using cAMP or forskolin reduces  $\gamma_j$  of hCx43 channels [75], but has no effect on rCx43 channels [11]. In many (but not all [78]) reports on PKA stimulation in rat and hamster cardiomyocytes, an increase of  $g_j$  is found upon application of cAMP [77, 91, 92], isoproterenol [92, 93], forskolin [92], norepinephrine [94], or by dialyzing the catalytic subunit of PKA into the cells [95]. Comparison of data from transfected cells with data from cardiomyocytes to explain differences is hindered by the fact that no  $\gamma_j$  values are available from studies on cardiomyocytes.

Also tyrosine phosphorylation reduces  $g_j$  which can be induced by inhibition of tyrosinephosphatase, or by activation of viral tyrosine kinases as p130<sup>gag-fps</sup> and v-Src [96, 97]. This is illustrated by a reduction of  $g_j$  when Cx43 is phosphorylated by v-Src on tyr265 [98]. Besides the tyrosine kinase activity of v-Src, downstream activation of MAP kinase induces phosphorylation on serine residues [99].

Generally, in cardiomyocytes, most pathways leading to phosphorylation of Cx43 seem to reduce junctional communication. When compared to transfected



**Fig. 4.** Western blot showing mouse Cx40 separated on 10% SDS-PAGE. HeLa cells overexpressing mCx40 were either unstimulated or stimulated for 10 min with TPA or 4 $\alpha$ -PDD. Using a polyclonal anti-Cx40 antibody from Alpha-Diagnostics, we were able to show a mobility shift of Cx40 which revealed a second band of approximately 42 kDa. The non-phosphorylating structural analogue of TPA, 4 $\alpha$ -PDD, did not induce this shift, thereby indicating that the 42-kDa band represented a phosphorylated form of the protein.

cells, some discrepancy exists regarding the modulatory role of stimulated PKC. Obviously, the presence and function of multiple protein phosphatases and their regulation by activated protein kinases have to be included in our understanding how cellular signals involved in modulation of communication are handled within the cell. In addition, the stimulation approaches used in the experimental studies are rather coarse when compared with the sophisticated interplay between cellular signal transduction pathways.

#### B2: Phosphorylation of Cx37, Cx40 and Cx45 Gap Junctions

Although much less is known about phosphorylation of cardiac connexins other than Cx43, several interesting data are present. Western analysis showed that Cx40 exists in a phosphorylated and nonphosphorylated configuration [3, 6]. In HeLa cells transfected with mCx40, direct activation of both PKC and PKA increased phosphorylation of Cx40 (measured by incorporation of  $^{32}\text{P}$ ), but did not alter electrophoretic mobility of the 40-kDa native protein on Western blot [3]. In our hands, using a nonradioactive approach we were able to show that stimulation of these HeLa cells with TPA (activation of PKC and MAP kinase) induces a shift in mobility of mCx40 on a Western blot whereas the nonstimulating structural analogue 4 $\alpha$ -PDD did not (see fig. 4). Similarly, in hCx40 transfected cells, a shift from 40 to 42 kDa was found after stimulation of PKA [6]. In addition,  $g_j$ ,  $\gamma_j$  and dye permeability increased significantly [6]. Until now, no modulatory role for activated PKG has been reported.

Cx37 also serves as a target for phosphorylation. FLAG-tagged human Cx37, introduced in BWEM cells, was phosphorylated on serine residues and upon phosphorylation the protein migrated on Western blot at 38 kDa instead of 36 kDa [100].

Cx45 is phosphorylated mainly on serine-, but also on tyrosine residues [18, 101–103]. Phosphorylation of serine residues in the carboxy tail of mCx45

prolongs the half-life of the protein [104]. There is debate on the electrophoretic mobility of the protein. Some groups have separated Cx45 as a single band of 45 kDa [104, 105], or of 48 kDa [16], while others claim to have detected two bands of 46 and 48 kDa, of which the 46-kDa band was proposed to be either a proteolytic product [103], or the unphosphorylated state of the native protein [18]. We reported that  $\gamma_j$  of hCx45 channels decreased upon stimulation of PKC while PKA and PKG appeared ineffective. Phosphorylation through activation of PKC elicits a third conductance state of 16 pS [11]. In contrast,  $\gamma_j$  of mCx45 channels is neither affected by stimulation of PKC, PKA, PKG nor by tyrosine phosphatase inhibition mediated by pervanadate. However,  $g_j$  decreases upon stimulation of PKA and inhibition of tyrosine phosphatase, but increases upon stimulation of PKC. Changes in  $g_j$  upon stimulation of PKA and phosphatase inhibition were accompanied by an increase in phosphorylation as shown on Western blot [18].

The generalized effects of phosphorylation are summarized in table 1.

### *Alterations in Expression of Gap Junction Proteins*

Apart from alteration of single channel conductance, alterations in number of expressed channels influences intercellular communication. In transfected cells, expression of connexins is controlled by an artificial promoter. Thus, using such cell systems only provides relevant information about modulation of properties during short-term processes if we want to compare this with processes in the natural cardiac environment. Studies on changes in expression and distribution of cardiac connexins as induced by prolonged stimulation of protein kinases or by application of growth factors, are generally performed using cultured neonatal (1- to 2-day-old) rat ventricular myocytes.

Mechanical stretch of such cultures activates multiple signalling pathways and has been reported to strongly increase expression of Cx43 but not of Cx40 [106, 107]. Increased expression of Cx43 resulted both from an increase in mRNA and a decrease in turn-over of the protein [107]. Myotrophin, strongly increased the amount of Cx43 mRNA [108], while cAMP significantly increased conduction velocity in strands of cultured myocytes [102]. This latter effect was explained by increased expression (gap junctions increased in number and size) of Cx43 and Cx45 which resulted from an increase in Cx43 mRNA and an enhanced translation of Cx45 mRNA. A comparable increase in Cx43 expression (functionally shown by more and larger gap junctions) was observed in an AT-1-receptor-mediated response after stimulating cells with Ang-II [109]. Wnt-1 stimulation of neonatal rat cardiomyocytes increased Cx43 mRNA and protein, which resulted in enhanced dye permeability and propagation



**Table 1.** Generalized effects of phosphorylation on cardiac gap junction channel characteristics

	PKA $g_j$	PKA $\gamma_j$	PKA dye coupling	PKC $g_j$	PKC $\gamma_j$	PKC dye coupling	PKG $g_j$	PKG $\gamma_j$	PKG dye coupling
Cx40	▲[6]	▲[6]	▲[6]	ND	ND	ND	ND	ND	ND
Cx43	▲[77, 91, 95] = [78]	▼[75] = [11]	= [11]	= [79] ▼[80, 85] ▲[11, 78, 88]	▼[11, 75, 78] = [80]	▼[11, 78, 86] = [79]	▼[11, 77, 78] = [74]	▼[11,77,78] = [74]	▼[11, 77, 78] = [74]
Cx45	▼[18]	= [11, 18]	ND	▲[18]	▼[11] = [18]	ND	= [18]	= [11,18]	ND

Overview of the effects of short-term stimulation of PKA, PKC and PKG on the total electrical conductance ( $g_j$ ), single channel conductance ( $\gamma_j$ ) and dye permeability of Cx40, Cx43 and Cx45 gap junction channels. Effects on Cx37 channels are lacking (ND). ▲ = Increase; ▼ = decrease; = = no effect; ND = not done. Contradictory effects may be caused by differences in the origin of the isoform studied (which species, e.g. [74]), experimental approach, dye molecule used or protein kinase isoform involved [e.g. 77, 78, 79].

of calcium [110]. In nonmyocyte cardiac cells like fibroblasts, bFGF increased both Cx43 mRNA and protein thereby increasing dye transfer [111]. In human umbilical vein endothelial cells (HUVEC), van Rijen et al. [112] showed that 48 h of exposure to tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) strongly impaired expression of Cx37 and Cx40 while Cx43 expression remained at control level though the channels seemed redistributed. Concomitantly, dye transfer of LY was impaired. These effects appeared to be reversible upon withdrawal of TNF- $\alpha$ . The temporal uncoupling of endothelial cells upon exposure to a proinflammatory cytokine such as TNF- $\alpha$ , might physiologically contribute to increase permeability of the endothelium thereby facilitating the migrating of cells participating in the inflammatory response. Other potentially interesting factors as epidermal growth factor (EGF), platelet-derived growth factor (PDGF), transforming growth factor  $\beta$  (TGF- $\beta$ ), and interleukin 1 $\beta$  (IL-1 $\beta$ ), also have been shown to affect Cx43 in noncardiac cells [113–116].

### **Concluding Remarks**

In this chapter, we have provided an overview about the current status of knowledge on physiology of the cardiac gap junction channels composed of Cx37 (endothelium), Cx40, Cx43 and Cx45 (cardiomyocytes). Though information is obtained from experiments performed at a reduced level of complexity, this undeniably helps to understand intercellular communication in the intact heart. The use of noncommunicating tumor cells transfected with a particular connexin isoform revealed that gap junction channels composed of different connexin isoforms have different properties and are differently regulated. The observations that the isoforms are not uniformly expressed throughout the heart but appear in a regional patterning suggests a functional reason for this diversity in expressed isoforms. This is even more stressed by changes which occur during development of the heart where, in time, fluctuations in expression levels must have a functional reason. The strength of the experiments with transfected cells is obvious since results are highly comparable to those obtained from experiments using isolated and cultured cardiomyocytes.

The information acquired from molecular biology and single channel behavior certainly helped to understand how gap junctions participate in impulse propagation and endothelial function under normal and pathological conditions. In the intact heart, the contribution of the involved isoforms has been addressed by germline gene deletion using genetically engineered mouse models. In this way, disruption of Cx40 expression revealed a reduced conduction velocity in the atria and ventricular conduction system, and an enhanced

propensity to develop arrhythmias [117, 118]. To test whether one isoform could be replaced by another without loss of function, the coding region of Cx40 was replaced by that of Cx45. This strategy confirmed the functional importance of isoform-specific properties as the normal sequence of impulse propagation could not be rescued in this model [119]. Deletion of Cx43 and Cx45 initially resulted in nonviable phenotypes due to developmentally induced malformations [120–122]. These cardiac and vascular malformations become more pronounced when combinations of isoforms are knocked out simultaneously (e.g. Cx40/Cx43 and Cx37/Cx40) [123]. To circumvent the occurrence of developmental defects, inducible knock-out strategies have been developed. Inducible deletion of Cx43 from adult hearts reduced, as expected, ventricular conduction velocity, and triggered the onset of ventricular tachyarrhythmias and sudden death [124]. For Cx40 and Cx45, this approach still has to be reported. In the endothelium, single deletion of Cx43, Cx40 or Cx37 did not result in striking alterations in endothelial/vascular function but did reveal that deletion of one isoform negatively affected expression of the other [125].

However, also less radical alterations in junctional conductance seem to reflect on cardiac impulse propagation. Regional downregulation of Cx43 expression by disease or infarction disrupts the natural pattern of conduction. Under conditions of ischemia, dephosphorylation of Cx43 [126], along with elevated levels of intracellular  $\text{Ca}^{2+}$  and  $\text{H}^+$ , might contribute to the observed reduced conduction velocity which can be explained by the effects of these parameters on single channel behavior. Based on the described experiments with isolated cardiomyocytes, we can also understand how high levels of circulating catecholamines, which are commonly present in several forms of hypertrophic cardiomyopathy, as well as growth factors, second messengers and activation of aberrant signal transduction pathways, contribute to the maladaptive changes in expression and function of the gap junction channels.

Future research will further evaluate the modifying role of these factors present in cardiac disease. If the mechanisms behind alterations in channel expression and performance can be elucidated using reduced levels of complexity, this will certainly contribute to our understanding of disturbed impulse propagation in the intact heart and facilitate development of intervention strategies in the future.

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## Role of Connexin43-Interacting Proteins at Gap Junctions

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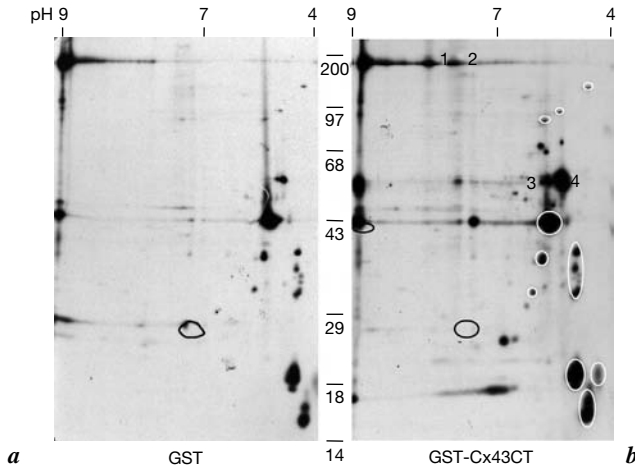
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### Abstract

Gap junctions are arrays of cell-to-cell channels that allow diffusion of small molecules between neighboring cells. The individual channels are formed by the four-transmembrane connexin (Cx) proteins. Recently, multiple proteins have been found to interact at the cytoplasmic site with the most abundant connexin, Cx43, but physiological data about the role of these interactions is scarce. Here, molecular detail about Cx43 interactions is presented and the putative roles of Cx43-interacting proteins are discussed. Emphasis is on new insights into the interactions of c-Src and ZO-1 with Cx43, interacting proteins discovered within the last 2 years (drebrin, CIP85, CCN3), and feedback between gap junctions, adherens junctions (N-cadherin and catenins) and the cytoskeleton (microtubules and actin).

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Gap junctional communication (GJC) between neighboring cells in tissues enables transfer of signaling molecules, metabolites and the spread of action potentials [1]. One gap junction channel consists of 12 integral membrane proteins of the connexin family [2, 3]. Twenty-one connexin isoforms have been identified in humans [4], of which 3 are found in the heart: Cx40, Cx43 and Cx45 [5]. Cx43 is the first identified connexin, the major connexin in myocardium and several other tissues, and it is the most ubiquitously expressed connexin. Cx43 gating is regulated by several mechanisms and the C-terminal tail of Cx43 contains multiple protein-protein interaction motifs and target sites for protein kinases [5–9]. Recent evidence suggests that, in addition to their well-established function as channel-forming proteins, connexins might have functions independent of GJC [10]. These channel-independent effects, implicated in control of cell migration and proliferation, might be mediated via Cx43 interactions with other proteins.



**Fig. 1.** Analysis of Cx43-interacting proteins. Lysates from  $^{35}\text{S}$ -Met/Cys-labeled Rat-1 cells were subjected to pull-down assays using GST only as a control (**a**) or fused to the Cx43 C-terminal tail (**b**) coupled to glutathione-Sepharose beads. Precipitated proteins were eluted and analyzed by two-dimensional SDS-PAGE/iso-electrical focussing. The most prominent GST-binding proteins (**a**) are encircled (white) in the GST-Cx43CT autoradiogram (**b**) and should be disregarded as nonspecific. Likely protein identity: 1, 2: phospho-ZO-1 (predicted MW 195 kDa, IEP nonphosphorylated ZO-1 is 6.3); 3:  $\alpha$ -tubulin (50 kDa/IEP:4.9); 4:  $\beta$ -tubulin (50 kDa/IEP:4.8); Black circles represent marker proteins (GST 27 kDa/IEP:6.6; actin 43 kDa/IEP:5.2). IEP: predicted iso-electric point; kDa: predicted molecular weight in kilodaltons (both calculated at [http://us.expasy.org/tools/pi\\_tool.html](http://us.expasy.org/tools/pi_tool.html)). Reproduced from Giepmans et al. [16].

Biochemical and molecular biology-based screening methods have been utilized to identify Cx43-interacting proteins that might be involved in regulation of gap junction synthesis and degradation, gap junction gating and Cx43-mediated channel-independent effects. GST pull-down assays (fig. 1) and yeast two-hybrid approaches using the isolated carboxy-terminal tail of Cx43 identified Src [11–13], ZO-1 [14, 15], microtubules [16], CCN3 [17, 18] and CIP85 [19] as interacting proteins (fig. 2, tables 1, 2). Using colocalization and coimmunoprecipitation, Cx43 has been shown to associate with several additional proteins, e.g. adherens junction proteins [20 and references therein] and cytoskeletal proteins [21]. Because most Cx43 partners have been found in protein-protein interaction screens (fig. 1), the physical interaction is often understood in detail, but the precise function is still more elusive.

The current chapter updates on Cx43-interacting proteins recently described in detailed reviews [5–9]. The function that these Cx43-binding

proteins might play in gap junction assembly, gating, turnover and the effect on adherens junctions, the cytoskeleton and gene regulation will be discussed.

### **Gap Junction Trafficking, Gating and Turnover**

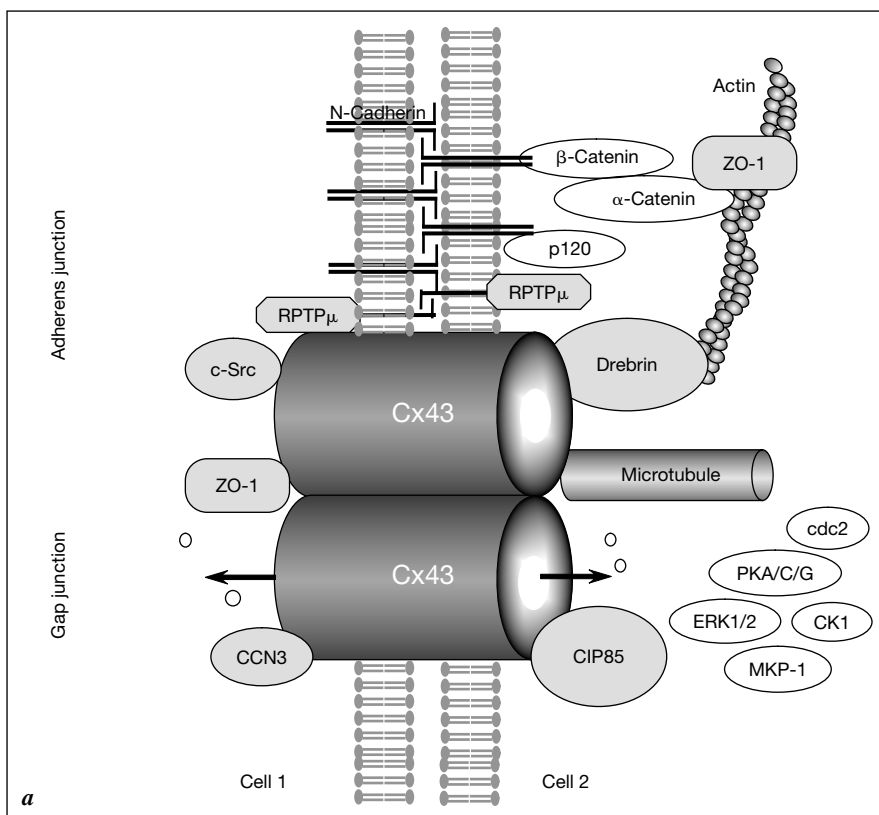
Compared to most structural proteins, Cx43 has a short half-life (only a few hours) and thus regulation of turnover of Cx43 is an important means of controlling GJC. Following synthesis of the four-transmembrane Cx43 protein in the endoplasmic reticulum, six monomers assemble into half a channel (connexon) in the Golgi followed by transport to the plasma membrane. During this assembly and transport Cx43 is being phosphorylated. At the plasma membrane, connexons diffuse lateral and two connexons of opposing cells dock and fuse at the edge of existing gap junction plaques or form new gap junctions. Cx43 channel activity is regulated by numerous conditions, including voltage, pH, oncogenes, protein kinases and growth factors [5–9]. Degradation of channels can occur through internalization of connexins from the center of the plaque, or internalization of a complete gap junction into one cell, giving rise to annular junctions [for protein interactions during transport, see ref. 8, 9].

#### *Regulation by Serine/Threonine Kinases*

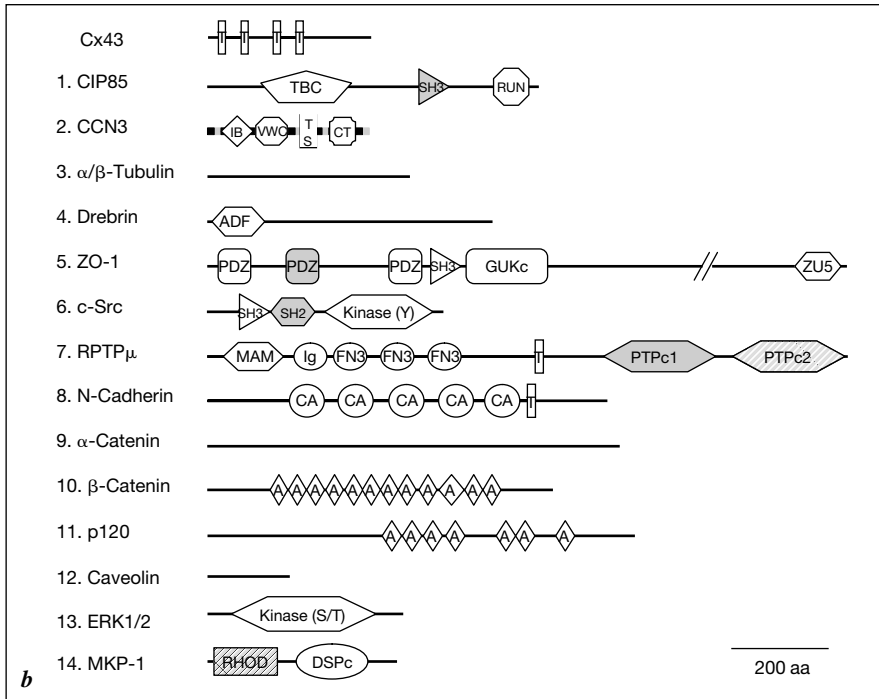
Numerous studies have established the importance of certain protein kinases in Cx43 phosphorylation for proper channel assembly, plasma membrane insertion and both inhibitory and stimulatory effects on GJC [recently reviewed in ref. 5–9]. Physical interactions between Cx43 and most protein kinases or phosphatases are not commonly found, which might reflect a transient or indirect association. An example of transient association is increased association of PKC $\epsilon$  and Cx43 in cardiac myocytes induced by fibroblast growth factor-2 (FGF-2), which correlates with Cx43 phosphorylation and inhibition of GJC. Whether PKC $\epsilon$  only acts downstream of FGF-2 or plays a more general role in inhibition of GJC and the mechanism of reduced gating remains to be explored [22].

#### *c-Src, ZO-1, MAPK and MKP-1 at Gap Junctions during Acidosis and Hypoxia*

Src and ZO-1 are the best-characterized Cx43-binding proteins (table 1). Binding of Src to Cx43 involves binding of the SH3 domain of Src to a proline-rich region of Cx43, tyrosine phosphorylation of Cx43, and binding of its SH2 domain resulting in channel closure [11–13] (table 1). ZO-1 binding to Cx43 is



**Fig. 2.** Proteins at Cx43-gap junctions. **a** Schematic view of protein-protein interactions at Cx43 gap junctions. Cx43 has been found to coprecipitate with several adherens junction proteins, namely N-cadherin,  $\alpha/\beta$  catenin and p120. This might reflect a physical association between these two junctions during cell-to-cell contact formation. Other proteins reported at both adherens and gap junctions, but not reported to be in the same complex are ZO-1, c-Src and RPTP $\mu$ . Recently, drebrin was reported to associate with Cx43 and link Cx43 to the actin cytoskeleton, which might increase gap junction stability. An opposite role has been suggested for CIP85, which binds via its SH3 domain to a proline-rich sequence in Cx43. CCN3 expression is induced by ectopic expression of Cx43 and has been suggested to regulate proliferation. Several kinases that (in)directly phosphorylate Cx43 are depicted at the lower right. These kinases have not been found to bind Cx43 directly, which might reflect the transient or indirect action of these kinases on Cx43. cdc2: Cell division cycle 2; CK1: casein kinase 1; PKA/C/G: protein kinases A/C/G. See table 1 for remaining abbreviations and see text for further details. Modified from Giepmans [7]. **b** Predicted protein modules in Cx43-interacting proteins. Cx43 is shown as a reference. 1–6: Shown to bind Cx43 directly; 5–11: proteins reported at adherens junctions. T = Transmembrane domain; A = ARM domain; TS = Tsp1 domain. Gray boxes: mapped Cx43-interacting domains. Scale in amino acids (ZO-1 is 1,736 amino acids). Gray lines = typically extracellular location; black



mediated by a PDZ domain/C terminal tail interaction [14, 15], and binding of c-Src and ZO-1 to Cx43 has been reported to be mutually exclusive [12].

Recently, Spray and coworkers addressed whether association of c-Src and ZO-1 with Cx43 is altered during two conditions when GJC is inhibited: intracellular acidosis and chemical ischemia/hypoxia, an in vitro model for ischemia in vivo [21, 23 and references therein]. The effect of pH on Src/ZO-1 association with Cx43 was addressed in primary astrocytes, which primarily express Cx43. Acidification (pH 7.4 versus 6.5) dramatically decreased GJC, and was accompanied by a redistribution of Cx43 from the plasma membrane to cytoplasmic compartments, whereas ZO-1 remained juxtamembrane. Furthermore, coimmunoprecipitation of Cx43 and ZO-1 was lost at pH 6.5, while the association of Cx43 with c-Src was induced under these conditions [23]. The phosphorylation

lines = cytoplasmic localization. Note that CCN3 is found in both compartments. See for common abbreviations and background table 1 and 2. Protein modules were predicted for human proteins (except CIP85, mouse) using the 'Simple modular architecture research tool' (SMART; <http://smart.embl-heidelberg.de/>) [46].

**Table 1.** Protein partners of Cx43: basic characteristic and relation to gap junctions

1. CIP85 (Cx43-interacting protein 85 kDa; 85 kD; IEP:5.6)  
Recently cloned when found in yeast two-hybrid screen as a binding partner of the Cx43 carboxy-terminal tail. Ubiquitously expressed. Binding involves <sup>253</sup>PLSP<sup>256</sup> in Cx43 and the SH3 domain of CIP85. Suggested to induce Cx43 degradation [19]
2. CCN3 (Cyr61/connective tissue growth factor-3, nephroblastoma overexpressed; 39 kDa; IEP: 8.2)  
Also known as NOV. Secreted protein with anti-proliferative effects [43]. Upregulated by ectopic Cx43 expression and localizes at the cytoplasmic site of Cx43 gap junctions. Implicated in anti-proliferative effects independent of GJC [17, 18]
3.  $\alpha/\beta$ -Tubulin [both 50 kDa; IEP:4.9 ( $\alpha$ )/4.8 ( $\beta$ )]  
Form dimers that constitute microtubules. Microtubule dynamics have been suggested to be down-regulated by cell–cell junctions [41]. A subset of microtubule ends binds directly to and colocalizes with a subset of Cx43-based gap junctions. Suggested to regulate microtubule stability at cell-cell contacts [16]
4. Drebrin (71 kDa; IEP:4.4)  
Actin-binding protein highly expressed in brain, but also found in other tissues. Involved in neurite outgrowth [47]. Links Cx43 gap junctions to actin, which might stabilize Cx43 gap junctions [25]
5. ZO-1 (zona occludens-1 protein; 195 kDa; IEP:6.3)  
Found at tight junctions, adherens junctions and gap junctions [7]. Might link cell-cell junctions to the actin cytoskeleton [26, 27]. Binds to the extreme Cx43 C-terminal tail by a PDZ2 interaction [15, 24], which can be inhibited by c-Src binding to Cx43 [12, 23, 24]. Function is not clear, but implicated in regulation of gap junction size, internalization [29, 48] and linkage to the cytoskeleton (see ‘Adherens Junctions Meet Gap Junctions: Junction Formation and Cell Migration’)
6. Src (60 kDa; IEP:7.1)  
Tyrosine kinase involved in numerous processes. Active Src can phosphorylate and bind Cx43 leading to gap junction closure (see ‘c-Src, ZO-1, MAPK and MKP-1 at Gap Junctions during Acidosis and Hypoxia’). Intermediate in receptor-mediated gap junction closure (reviewed in [5–9])
7. RPTP $\mu$  (receptor protein tyrosine phosphatase  $\mu$ ; 164 kDa; IEP:6.2)  
Transmembrane tyrosine phosphatase found at cell-cell contacts. Found to co-immunoprecipitate with Cx43 via its catalytic domain. Inhibition of tyrosine phosphatases correlates with gap junction closure. Proposed to counteract Src function [49]
8. N-cadherin (99 kDa; IEP:4.7)  
One of the basic integral membrane proteins that forms adherens junctions. Found to coprecipitate and colocalize with Cx43. Interaction with Cx43 has been implicated in formation of gap junctions and adherens junctions [20, 35].
9.  $\alpha$ -Catenin (100 kDa; IEP:6.0)  
Adherens junction protein implicated in cell adhesion and regulating actin dynamics [38]. Found to colocalize and coprecipitate with Cx43 [20, 37] and thought to play a role in Cx43 trafficking
10.  $\beta$ -Catenin (85 kD; IEP:5.5)  
Adherens junction protein that binds multiple proteins and forms a link to the cytoskeleton. Also a transcriptional co-factor in the Wnt pathway. Found to coprecipitate and colocalize with Cx43 [20, 35, 50], which might reflect coformation of adherens junctions and gap junctions [20]
11. p120 (p120 catenin; 108 kDa; IEP:5.9)  
Adherens junction associated protein, implicated in regulation of cadherin turnover and motility [39]. Found to coprecipitate and colocalize with Cx43 [20, 35, 37]. Association with Cx43 might reflect coformation of adherens junction and gap junctions, but is not crucial for gap junction formation [20]

**Table 1.** (continued)

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12. Caveolin (20 kDa; IEP:5.6)  
Constituted of certain lipid rafts (caveolae). Associated with concentration of proteins and lipids involved in transmembrane signaling. Found to colocalize, copurify and coimmunoprecipitate with Cx43 [51]. Suggests gap junctions associate with lipid rafts
  13. ERK1/2 (Extracellular regulated kinases 1 and 2; 43 kDa; IEP:6.3 (1); 41 kDa; IEP:6.5)  
Ser/ Thr kinases with several substrates. Translocates to the nucleus when activated and induces gene transcription leading to proliferation. Found to coprecipitate with Cx43 during CIH [21]. Function of this interaction is not known. ERK1/2 activity has been shown to phosphorylate Cx43 leading to both upregulation and inhibition of GJC (reviewed in [5–9])
  14. MKP-1 (dual specificity phosphatase 1/MAPK-phosphatase 1; 39 kDa; IEP:6.8)  
Dephosphorylates and thereby inactivates MAPK. Found to coprecipitate with Cx43 during chemical ischemia/hypoxia. Function unknown but proposed as a candidate phosphatase for Cx43 during chemical ischemia/hypoxia [21]
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**Table 2.** Domains in Cx43-interacting proteins

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ADF	Actin depolymerization factor/cofilin-like domain. Involved in actin remodeling by binding to actin monomers as well as f-actin
ARM	Armadillo/ $\beta$ -catenin-like repeats. Involved in protein-protein interactions at adherens junctions and in the Wnt pathway
CA	Cadherin repeats. Extracellular domains in cadherin. Involved in calcium-dependent adherens junction formation
CT	C-terminal cysteine knot-like domain (CTCK). Main homology is the conservation of cysteines. Predicted to form homodimers. Found in several growth factors
DSPc	Ser/Thr and Tyr dual specificity phosphatase catalytic domain. Enzymes containing these domains regulate mitogenic signaling and the cell cycle
FN3	Fibronectin type 3 domain. Extracellular domain interacts with proteins and other molecules. Implicated in numerous processes, including cell adhesion, cell migration and cytoskeletal maintenance
GUKc	Homologue to yeast guanylate kinase. Found in membrane-associated kinases. No reported kinase activity in higher eukaryotes, but can mediate protein-protein interactions
IB	Insulin growth factor binding domain. Binds insulin-like growth factors in the extracellular space and affects their signaling
Ig	Immunoglobulin. Extracellular globular domain involved in interactions with proteins and other molecules
Kinase	Key switch in signal transduction by phosphorylation of proteins. Specific for tyrosine (Y) or serine/threonine (S/T) residues
MAM	Found in extracellular domain of certain transmembrane proteins. Likely mediating adhesion by homodimerization via disulfide bridges
PDZ	PSD-95/discs large/ZO-1 homology domain. Binds proteins via dimerization, internal binding, or binding to the C-terminal hydrophobic residue of mainly plasma membrane (associated) proteins in its hydrophobic pocket

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**Table 2.** (continued)

PTPc1	Protein tyrosine phosphatase catalytic domain. Counteracts tyrosine kinase function
PTPc2	Highly homologous to PTPc1, but lacking phosphatase activity
RHOD	Rhodanes homology domain. The enzymatically inactive domain in MKP-1 likely plays a role in protein-protein interactions
RUN	Found in several proteins functionally linked to Rab and Rap GTPases. Function unknown.
SH2	Src Homology 2 domain. Binds phospho-tyrosine residues in a hydrophobic pocket. An additional pocket confers substrate specificity by binding to more C-terminal amino acids
SH3	Src Homology 3 domain mediates protein interactions by binding to proline residues located at one side of an $\alpha$ -helix in its hydrophobic pocket
TBC	Homologous to GTPase-activating proteins in yeast and putative activating proteins for Rab-like GTPases in higher eukaryotes
TSP1	Thrombospondin type I repeats. Found in secreted proteins. Involved in cell-cell interaction, inhibition of angiogenesis and apoptosis
VWC	von Willebrand factor type C domain. Found in secreted proteins, but also nuclear. Implicated in formation of multi-protein complexes and regulation of numerous processes including cell adhesion and migration
ZU5	Present in ZO-1 and Unc-5 netrin receptors, as well as some ankyrins that attach integral membrane proteins to the cytoskeleton. Function unknown

Descriptions have been modified from SMART (<http://smart.embl-heidelberg.de/>) [46].

state of Cx43 was found to be dramatically affected under conditions of chemical ischaemia/hypoxia: tyrosine phosphorylation of Cx43 was induced, presumably mediated by c-Src, whereas Ser/Thr phosphorylation seemed to be diminished [21]. Besides c-Src, extracellularly regulated kinase (ERK1/2; table 1) and MAPK phosphatase-1 (MKP-1; table 1) were found to coimmunoprecipitate with Cx43 during chemical ischemia/hypoxia, but not under control conditions, while the association of Cx43 with actin (see ‘Actin’ below) was decreased during chemical ischemia/hypoxia [21]. The molecular details of ERK1/2 and MKP-1 associations with Cx43 (direct or indirect), their possible effect on the phosphorylation state of Cx43 and the function of these proteins during chemical ischemia/hypoxia remain to be established.

Detailed molecular insight has been obtained for the Cx43 interactions with Src and ZO-1 using isolated domains in vitro. Resonant mirror spectroscopy confirmed that the PDZ2 domain of ZO-1, but not PDZ1 or PDZ3, mediates the interaction [23], in agreement with previous studies [14, 15]. However, PDZ2 association with the C-terminal Cx43 tail did not alter by changing the pH from 7.4 to 6.5. Using the same approach, the SH3 domain of c-Src was found to bind more efficiently to the Cx43 tail at low pH. When the SH3 domain of c-Src is bound to Cx43, the ZO-1 PDZ2 domain binding to this

Cx43 tail complex is inhibited [23]. Further molecular detail was obtained by NMR data: The PDZ2 domain of ZO-1 structurally modifies the C-terminal 19 amino acids of Cx43 [24], consistent with earlier reports that showed the importance of the C-terminal integrity for ZO-1 binding [12, 15]. Interestingly, the SH3 domain of c-Src was not only found to modify a previously identified proline-rich Src-binding domain in Cx43 [12], but also 4 more C-terminal located stretches of amino acids, including 1 within the carboxy-terminal 19 amino acids residues that was affected by PDZ2 binding. Moreover, the SH3 domain of c-Src was found to displace the ZO-1 PDZ2 domain bound to the Cx43 C-terminal tail in a competition assay [24]. These findings do not support an earlier model that suggested that a Src-SH2 domain binding to Cx43 was responsible for dissociation of ZO-1 [12]. The differences between these models might be explained by the techniques used: the *in vitro* studies are based on isolated protein modules, whereas the cell culture experiments use complete proteins and a cellular environment that adds a higher level of complexity. Alternatively, c-Src might act on Cx43 at different levels. Closure of Cx43 gap junction channels by e.g. certain growth factors seems to be dependent on tyrosine phosphorylation of Cx43 by c-Src followed by an SH2 domain interaction that can be rescued by Cx43-Tyr265Phe [7, 8]. Under conditions of acidification, however, c-Src might play a role in internalization of Cx43 involving the SH3 domain interaction and disruption of the Cx43/ZO-1 association [23, 24].

#### *CIP85: Increasing Lysozomal Degradation of Cx43?*

The SH3 domain of rab-like 85 kDa Cx43-interacting protein (CIP85; fig. 2, table 1) was identified as a Cx43-interacting protein in a yeast two-hybrid screen [19]. Subsequently, the complete cDNA of this protein with unknown function was cloned. Four modular domains are predicted in CIP85 (fig. 2b, table 2) and the gene is ubiquitously expressed. The association with Cx43 involves the SH3 domain of CIP85 and the <sup>253</sup>PLSP<sup>256</sup> motif in the carboxy-terminal tail of Cx43. Immunolocalization studies revealed that CIP85 was localized at gap junctions. Pulse-chase experiments showed increased degradation of Cx43 expressed in HeLa cells when CIP85 was overexpressed, but not in control cells or in cells expressing CIP85 lacking the SH3 domain that mediates Cx43 interaction, nor when lysozomal degradation was blocked [19]. Future experiments should give more insight in the function and colocalization of CIP85 with Cx43 and its role in Cx43 degradation.

#### *Drebrin: Stabilizing Cx43?*

The actin-binding protein drebrin (fig. 2b, table 1) was identified as a Cx43 partner in a proteomics screen [25]. Overexpression of C-terminal fluorescent protein-tagged drebrin and Cx43 revealed colocalization specifically at

gap junctions. While formally the interaction has not been shown to be direct, all the assays, including fluorescence resonance energy transfer (FRET) between fluorescent-protein-tagged drebrin and Cx43, are highly suggestive for a direct interaction. Knockdown of endogenous drebrin by RNAi decreased Cx43 expression, which mainly reduced the amount of gap junctional Cx43, and concomitantly decreased GJC. These results suggest that drebrin plays a role in maintaining Cx43 at the plasma membrane, possibly by preventing interaction with degradation machinery or by forming a highly structured complex between Cx43 and the cytoskeleton [25]. When the interaction sites are mapped, site-directed mutagenesis might further help to understand the role of drebrin at Cx43 gap junctions.

#### *ZO-1 at Gap Junctions: Multiple Functions?*

While activated Src leads to inhibition of GJC and may disrupt the ZO-1 interaction with Cx43 (outlined in ‘c-Src, ZO-1, MAPK and MKP-1 at Gap Junctions during Acidosis and Hypoxia’ above), the function of ZO-1 at gap junctions, but also at other cell-cell junctions, remains elusive. Over the past few years, several roles of ZO-1 at gap junctions have been postulated, which might reflect diverse functions of this scaffolding protein depending on cell type, presence of other cell-cell junctions. These putative functions are briefly discussed.

ZO-1 is the only Cx43-interacting protein reported at several other, but not all, connexin-isotype gap junctions [7, 8], as well as at other cell-cell junctions. Like other multi-PDZ containing proteins, ZO-1 is a candidate to cluster signaling molecules at these junctions. Via its actin-binding region, ZO-1 can link actin to tight junctions [26] and adherens junctions [27], which might play a role in junction assembly. Whether ZO-1 is involved in actin linkage to gap junctions remains to be established.

Internalization of gap junctions has been reported to correlate with an increase of the Cx43/ZO-1 association in dissociating cardiac myocytes, which have low basal levels of Cx43 complexed to ZO-1 [28]. Another suggestion that ZO-1 binding at gap junctions might be involved in regulation of gap junction turnover came from a study where C-terminal GFP-tagged Cx43, which cannot bind to ZO-1, was shown to form excessively large gap junction plaques [29]. Whether this plaque phenotype is indeed due to the incapability ZO-1 to bind tagged Cx43, or whether other Cx43-interacting partners are affected, or for instance dimerization of GFP affects plaque size, might be solved by a more subtle disruption of the ZO-1 binding site in Cx43. A reciprocal approach using the N-terminal half of ZO-1 as a ‘dominant negative’ suggests that competition with endogenous ZO-1 leads to a decrease of functional gap junctions, whereas over-expression of ZO-1 leads to increased GJC [30]. These experiments suggest that

gap junctions are stabilized by ZO-1 rather than degraded. However, interfering with ZO-1 might affect other cell–cell contacts or transcriptional regulation (see below). As for the Cx43-GFP approach, future studies with a subtler dominant-negative ZO-1 will help to pinpoint ZO-1 function at gap junctions.

Alternatively to ZO-1-regulating gap junctions, ZO-1 might be regulated downstream of gap junctions. Evidence for junctional regulation of ZO-1 came from studies on tight junctions, where ZO-1 has been shown to be able to sequester transcription factors and thereby regulate cell density and proliferation [31]. In analogy with tight junctions, one can envision that gap junctional ZO-1 sequesters transcription factors and thereby affects cell proliferation. Such a mechanism might explain channel-independent control of proliferation reported for Cx43 (see also ‘Growth Inhibition Mediated by Cx43: A Role for CCN3?’ below).

Potential redundancy by family members adds to the complexity of studying ZO-1 function. Recently, ZO-2 was reported to bind to the Cx43 tail *in vitro* [32]. Whether ZO-2 is found at gap junctions and the physiological role of this interaction remains to be established.

### **Adherens Junctions Meet Gap Junctions: Junction Formation and Cell Migration**

Adherens junctions and gap junctions have been known to feedback on each other for several years, and physical interactions between these junctions have been found. Several kinases and phosphatases involved in junction regulation have been identified in both junctions [reviewed in refs 5–8] (fig. 2a). As mentioned above, ZO-1 is prominent at both cell-cell junctions and has been shown to bind to connexins [14, 15], adherens junction proteins [27] as well as tight junction proteins [33]. ZO-1 is a candidate to physically link these junctions, but no experimental evidence is available to substantiate this notion.

A functional link between adherens junctions and gap junctions came from knockout studies by Huang et al. [34]. N-cadherin and Cx43 knockout neural crest cells both have migration defects. In normal neural crest cells, Cx43 colocalizes with several adherens junction proteins, such as N-cadherin and catenins (fig. 1, table 1). In the N-cadherin knockout cells, GJC is reduced, but cell surface Cx43 was not found to be changed [35]. In a follow-up study, using NIH3T3 cells, Cx43 was found to colocalize and coprecipitate with N-cadherin,  $\alpha$ -catenin,  $\beta$ -catenin, p120 and ZO-1 (fig. 2, table 1). This study, together with previous indications [20 and references therein], shows that Cx43 gap junctions can be in a macromolecular complex with N-cadherin adherens junctions, but the jigsaw puzzle of the precise interactions between individual proteins remains to be solved.

Functional cross-talk was further studied using RNAi knockdown. In Cx43 knockdown cells N-cadherin, as well as other typical adherens junction proteins, were no longer found at the plasma membrane. Interestingly, knockdown of p120 resulted in a loss of N-cadherin at cell-cell contacts, but Cx43-based gap junctions appeared normal, suggesting that Cx43 trafficking was not altered. Cell motility, as determined by cell shape changes and displacement, was reduced in N-cadherin and Cx43 knockdown NIH-3T3 cells compared to control cells [20], in agreement with defective migration found in neural crest cells of the knockout animals.

In N-cadherin knockdown cells, GJC was reduced and Cx43 at the plasma membrane was decreased, which was found to be caused by a trafficking defect, but Cx43 levels were unaltered [20]. Note the discrepancy with the results found in neural crest cells described above [35], as well as the observation that adherens junction proteins are not altered in cardiac myocytes of Cx43 knock-out mice [36]. As pointed out by the authors, this might reflect a cell-type- or context-dependent role for N-cadherin/Cx43 interaction in co-trafficking. In agreement with junction coassembly, Cx43 trafficking was previously found to depend on a preexisting catenin-ZO-1-Cx43 complex in cardiac myocytes [37].

In conclusion, depending on cell type and context, adherens junction and gap junction formation can be mutually affected, which seems to involve regulation of cell migration. While the exact mechanisms are not known,  $\alpha$ -catenin and p120 recently have been shown to regulate cytoskeletal dynamics [38, 39], and physical interactions between gap junctions and the cytoskeleton might be involved.

## **Gap Junctions and the Cytoskeleton**

Several drugs that disrupt or stabilize the actin and/or microtubule cytoskeleton have been shown to interfere with gap junction function. However, cell function is affected at numerous levels by these drugs, and therefore a direct causal relationship between the cytoskeleton and gap junction function is often unclear. Direct physical interactions between gap junctions and the cytoskeleton are discussed below.

### *Microtubules*

Electron microscopy studies have reported the presence of connexins, gap junctions and internalized gap junctions (annular junctions) close to cytoskeletal structures. Recently,  $\alpha$ - and  $\beta$ -tubulin were found to bind to Cx43 in pull-down assays (fig. 1) [16, 25]. Moreover, a subset of Cx43 gap junctions was found to colocalize with MT tips as determined by immunostainings at the light and electron microscopy levels [16, 40]. Short-term disruption of the

microtubule cytoskeleton seems not to be crucial for regulation of Cx43-based GJC [16]. Long-term (several hours) disruption of MTs by colchicine was found to increase cytoplasmic Cx43 staining, suggesting an increased turnover when microtubule-Cx43 interaction is disrupted [40]. Whether the Cx43-tubulin connection serves to control microtubules, a role that has been hypothesized for an unidentified cell-cell junction by [41], or microtubules control Cx43 gap junctions remains to be established.

### *Actin*

Actin might be linked to gap junctions by drebrin and thereby stabilize Cx43 at the plasma membrane (see ‘Drebrin: Stabilizing Cx43?’ above). A role for ZO-1 in linking the actin cytoskeleton and gap junctions (see ‘ZO-1 at Gap Junctions: Multiple Functions?’ above) is supported by coprecipitation of both actin and ZO-1 with Cx43 in astrocytes under control conditions, but not during chemical ischemia/hypoxia when ZO-1 dissociates from Cx43 (see ‘c-Src, ZO-1, MAPK and MKP-1 at Gap Junctions during Acidosis and Hypoxia’ above) [21]. Future studies focusing on the subcellular architecture at gap junctions during chemical ischemia/hypoxia are required to further address the actin-Cx43 connection.

## **Growth Inhibition Mediated by Cx43: A Role for CCN3?**

Several studies have indicated that Cx43, likely via its C-terminal tail, might be involved in gene regulation and growth suppression independent of GJC [reviewed in ref. 10]. Gupta et al. [42] studied the effects of Cx43 expression on gene regulation by differential display between Cx43 transfected and control C6 glioma cells and found upregulation of CCN proteins (table 1). CCN proteins have multiple modular domains (table 2) and are involved in wound healing, angiogenesis and chondrogenesis [43].

CCN3 (fig. 2; table 1), a protein reported to have both intracellular and extracellular functions [43], was found to physically interact at the cytoplasmic face of Cx43, but not Cx32, Cx40 or C-terminal truncated Cx43 that all form functional gap junctions [18]. Similar results were reported using human malignant fibroblasts. Induced expression of Cx43, but not Cx40 or the truncation mutant of Cx43, reduced cell proliferation in culture and tumor growth when injected in mice. Moreover, overexpression of CCN3 reduced proliferation in these cells [17]. The need of the presence of the C-terminal tail of Cx43 to mediate anti-proliferative effects supports the model proposed in earlier studies that Cx43 can mediate signaling independent of channel function [10, 44]. Interestingly, CCN3 translocates in Cx43-overexpressing cells from the nucleus and cytoplasm to cell-cell contacts [17]. The authors suggest a model in which

an N-terminal truncated form of CCN3 localizes to the nucleus and leads to growth stimulation. Secreted or Cx43-bound CCN3 might have the opposite effect and inhibit proliferation [17, 18]. Several questions that remain are: If CCN3 translocation out of the nucleus is a means of regulating proliferation, what does the upregulation of the protein mean (why does Cx43 not shut down expression)? How does CCN3 localized at gap junctions alter gene transcription? Is CCN3 a transcription (co)factor? Can gap junctions act as a ‘magnetic bar’ to sequester transcription factors? In analogy with regulation of subcellular localization of transcription factors by cell-cell junctions involving ZO-1 (see ‘ZO-1 at Gap Junctions: Multiple Functions?’ above) [45], CCN3 is a prime candidate to mediate anti-proliferative effects downstream of Cx43 gap junctions.

### **Concluding Remarks**

The recent mapping of protein interactions with Cx43 aids in understanding the formation and regulation of gating Cx43-based gap junctions in molecular detail. Moreover, it has become clear that gap junctions can be connected to other subcellular structures and that gap junctions might have additional roles to their channel-forming function. A picture is now emerging where the gap junction, with six tails exposed per connexon and thousands of tails per gap junction plaque, binds diverse signalling and structural proteins and thus forms a highly specialized structure crucial for development and tissue maintenance. Identification of more interacting partners is likely (fig. 1). Together with studies that address the tissue and spatiotemporal specificity, the molecular mechanisms and the exact role(s) of these interactions at gap junctions, we will increase our understanding of the importance of these unique structures in cell biology.

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## Life Cycle of Connexins: Regulation of Connexin Synthesis and Degradation

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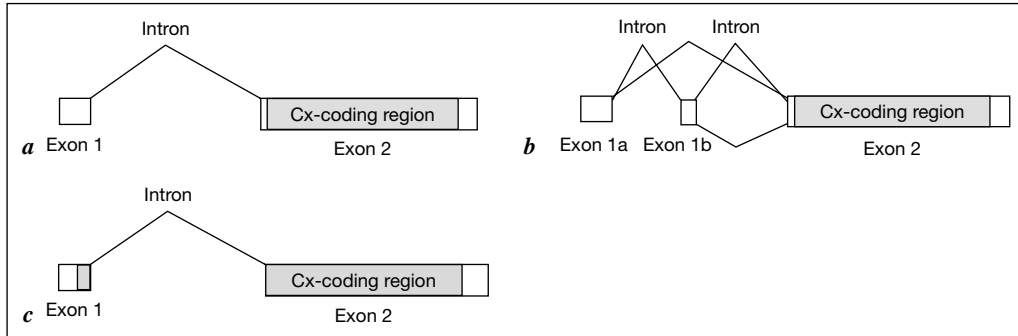
### Abstract

Gap-junction-forming connexins (Cx) exhibit a complex life cycle which is regulated at various levels. First, the promoter regions and binding of transcription factors to them control the transcription of the connexin genes. Translation of Cx-mRNA seems to be enabled by internal ribosome entry site elements allowing translation even under stress conditions. The newly synthesized Cx protein (monomeric) is transferred to the Golgi apparatus, oligomerized, transferred to the plasma membrane and incorporated into gap junction plaques. Two principal pathways for degradation of Cx could be defined: (a) lysosomal and (b) proteasomal degradation, including phosphorylation and ubiquitination as well as the internalization of complete gap junction channels as annular gap junctions doomed to degradation. In the present article, the various steps of the life cycle of cardiac connexins and its regulation are reviewed.

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‘Cardiac muscle cells communicate in life, but fail to give a message of their decay’ Engelmann [1] wrote in 1878 and although not being aware of the existence of gap junction proteins he characterized their key feature very clearly: the gap junction proteins composed of different types of connexins provide intercellular communication and cell coupling by forming dodecameric channels, thereby connecting neighboring cells. While gap junction channels are ubiquitous in all organs, they play a dominant role in cardiovascular tissue, particularly in the heart, where they constitute the biophysical basis of regular rhythm and conduction. Moreover, allowing an exchange of metabolites and small molecules up to 1,000 Da, for example the second messenger cAMP [2], the gap junction channels are capable of transferring chemical information to adjacent cells, thus synchronizing a cellular network.

In order to carry out these complex functions, it is essential that the expression of the gap junction protein (the connexin) and the degradation of the whole



**Fig. 1.** Configuration of connexin genes. Adapted from Söhl et al. [4]. **a** Unique splicing of one 5'-untranslated region. **b** Alternative splicing of 5'-untranslated region. **c** Splicing of the Cx coding region.

gap junction channel are precisely regulated and that different mediator molecules can interact with the regulation of connexin expression and degradation.

The following chapter will describe how connexins are synthesized, oligomerized, transferred to the cell membrane, incorporated into the membrane, how two hemichannels of adjacent cells find each other to form a complete pipe-like gap junction channel and finally how the gap junction channels are degraded. The regulation of degradation and the resulting rather short half-life of about 90 min will be considered as well.

## Synthesis of Connexins

In cardiac tissue, there are three main connexins Cx40, Cx43 and Cx45, which are important for a regular function of the heart, i.e. propagation of action potentials and muscle contraction. The genes encoding these connexins are located on chromosome 1 for Cx40, chromosome 6 for Cx43 and chromosome 17 for Cx45, respectively. The connexin gene itself has a relatively uniform structure: with a 5'-untranslated region, named exon 1, which is separated by an intron of variable length from exon 2 bearing the complete connexin-encoding region and the 3'-untranslated region [3, 4]. Starting from this basic structure, more detailed configurations of connexin genes have been proposed in the last years. In general, there are two main exceptions to this common structure: one involving different splicing variants of the 5'-untranslated region and the other, interruption of the connexin-encoding region itself by an intron [5, 6] (fig. 1; adapted from Söhl et al. [4]).

For the Cx43 gene, at least 6 different transcripts have been identified until now with three different promoter regions [5]. These promoter regions, named P1, P2 and P3, have variable binding sites for diverse transcription factors (i.e., Sp1, STAT factor binding sites were found in P3, a CREB-binding site and binding sites for c-Myc and HSP90 in P2 [7]. P1 is the dominant promoter and most commonly used for transcription and it is not clear why and for what specific purpose alternative promoter regions of the Cx43 gene are present. The two other promoters are perhaps only used in specific situations, for example cell differentiation or cell development.

Furthermore, it has been described that both transcription factors AP1 and Sp1 are necessary for optimal Cx43 promoter activity and that activation of PKC also leads to an upregulation of promoter activity through an AP1 site in the promoter sequence [8]. Besides these, there are other transcription factors (i.e., Nkx2.5 and Tbx2 and the Wnt-signaling pathway) which are involved in the transcriptional Cx43 regulation. Their role in up- or downregulation of Cx43 seems to be species and tissue specific and has to be further elucidated [9].

To make it even more complex, alternative splicing variants of the 5'-untranslated region have been reported and although knowledge of Cx43 gene regulation is still incomplete, it is obvious that different splicing variants may have an important effect on mRNA stability and/or mRNA translation. Analysis of the 5'-UTR region of Cx43 mRNA revealed an active IRES (internal ribosome entry site) element which is responsible for the translation efficacy of Cx43 mRNA. It has been shown that the IRES element is necessary for an efficient translation of connexin mRNA [10, 11]. Moreover, it has been shown that mutations in the IRES of connexins might lead to hereditary diseases as described for the IRES of the 5'-untranslated region of the Cx32 gene, which was found to be mutated in a family suffering from Charcot-Marie-Tooth disease [12].

The most common way of initiating translation in eukaryotic cells is the binding of the 40S ribosomal subunit to the 5'-cap structure of the mRNA. The 40S ribosomal subunit then scans the mRNA until it finds an AUG start codon which initiates association with the 60S ribosomal subunit thereby starting translation. Most of the mRNAs translated via this cap-mediated mechanism have relatively short 5'-untranslated regions (<50 nucleotides). In contrast, the 5'-untranslated region of various connexins are markedly longer (the 5'-untranslated region of Cx43 mRNA contains 208 nucleotides), which hinders cap-mediated translation. This raises the question of possible beneficial roles of this long 5'-untranslated region. It has been shown for several genes (i.e., transcription factors, heat-shock proteins and proteins involved in apoptosis) that their 5'-untranslated region harbors an IRES, which enables translation, even under conditions when cap-mediated translation is suppressed i.e. in cellular stress situations. Since connexins are essential for intercellular communication,

the IRES-mediated translation allows rapid initiation of protein synthesis in response to cellular signals even under circumstances when cap-mediated translation is switched off [13].

Recently, three exons have been identified (exon 1A, exon 1B and exon 2) in the human Cx40 gene with the coding region located on exon 2 and two transcripts with different 5'-untranslated regions [14]. The transcript exon 1A/exon 2 is the dominant transcript, whereas the transcript exon 1B/exon 2 is only expressed at low levels. Moreover, the expression of these two transcripts seems to be species and cell type specific and the exact role of these two transcripts has not been characterized yet. But it might be conceivable that the regulation of translation is carried out via different spliced exons as has been described for the neuronal nitric oxide synthase [15]. Like the Cx43 gene, the Cx40 gene contains several binding sites for transcription factors involved in Cx40 mRNA expression. On exon 1A, a binding site for the cardiac transcription factors Nkx2.5 and Tbx5 has been identified as well as binding sites for AP1 and Sp1 and Sp3, all of them playing a positive role in Cx40 mRNA expression. Furthermore, as in the Cx36 gene, the reading frame of the human Cx40.1 is interrupted by an intron (fig. 1c). The complete coding region is then restored by splicing, but whether the Cx40.1 gene represents a different transcription isoform remains to be elucidated.

For the Cx45 gene, three exons and two introns have been identified in the mouse genome; however, the exact promoter region has not yet been established [16]. The first two exons contain the 5'-untranslated regions whereas the third exon harbors the remaining 5'-UTR and the whole coding sequence. Several splice variants of the Cx45 gene are known. However, these are not equally expressed in the same tissue. Cx45 in cardiac tissue plays a role predominantly in early developmental stages. Although in these stages a number of growth factors, hormones and mediators regulate tissue differentiation and growth, and although it might be assumed that Cx45 may also be subject to such a regulation, at present only very little is known on Cx45 gene regulation and promoter functions; thus, we must await future studies on this subject.

### **Connexin Oligomerization and Assembly**

Synthesis and trafficking of connexins have been investigated intensively in the past years. However, most of the studies refer to Cx43 and it is not definitely known whether all connexins follow the same pathways (indeed, there is evidence that for the Cx26 the intracellular routing is different [17]. In consequence, the following paragraphs will consider predominantly Cx43 as the classical reference connexin.

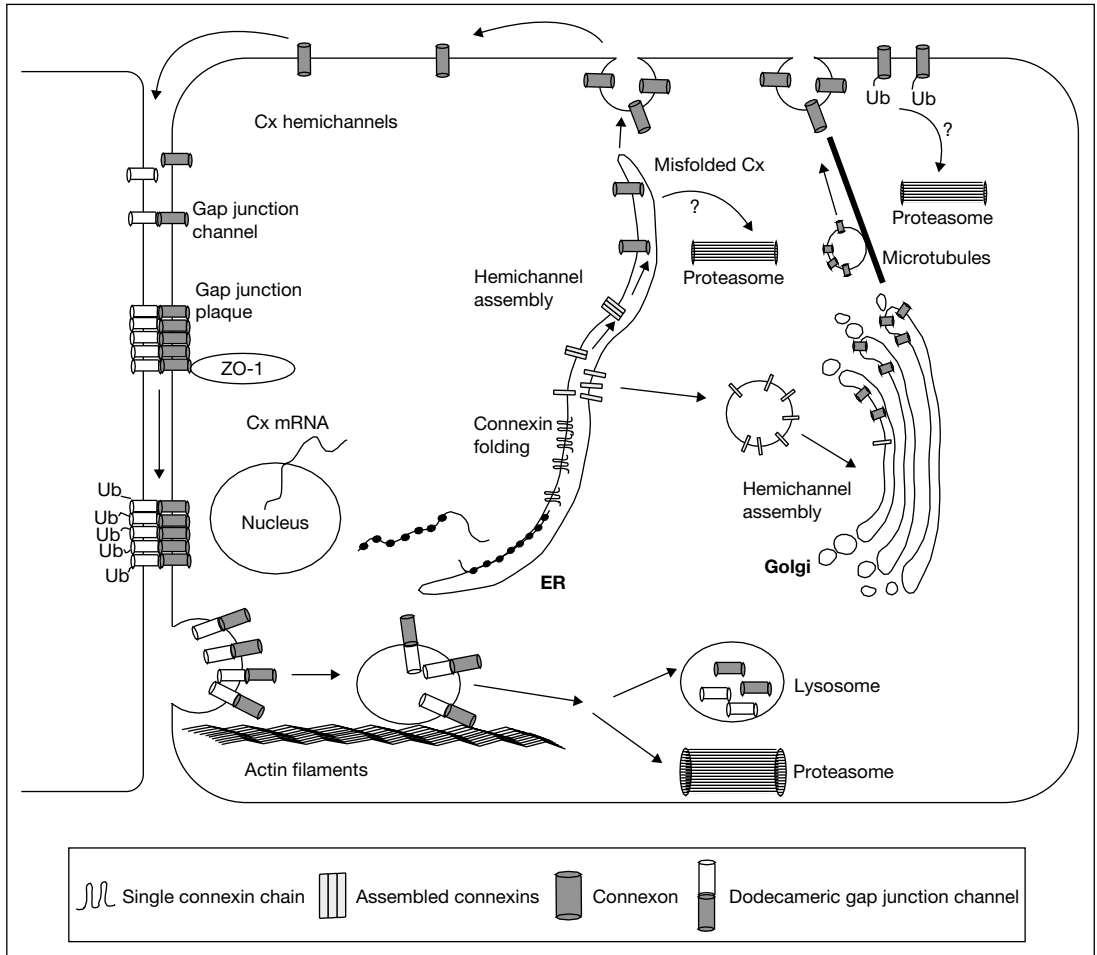
In general, synthesis, assembly, and trafficking of connexins largely appear to follow the general secretory pathway for membrane proteins: after transcription of the connexin mRNA, the protein is synthesized at the ribosomes of the endoplasmic reticulum (ER) and is cotranslationally inserted into the ER membrane, a process that requires an internal signal anchor sequence and docking of the connexin chain/ribosome complex to the translocon [18]. The final folding of the connexin peptide chain to its functional structure with four transmembrane domains, two extracellular loops and the N- and C-terminus located at the cytoplasmic side occurs during the integration into the ER membrane [19].

The nascent nonphosphorylated Cx43, initially translated as a 42-kDa polypeptide chain besides other steps, has to undergo several phosphorylation events to mature. The majority of phosphorylation steps occurs at serine residues and the resulting Cx43 isoforms with slower migrating abilities in SDS-PAGE are designated as P<sub>1</sub> (about 44-kDa), P<sub>2</sub> (about 46-kDa) and P<sub>3</sub> [20–22]. It is noteworthy that the P<sub>1</sub>/P<sub>2</sub>/P<sub>3</sub> nomenclature does not indicate the number of phosphate residues added to the nascent Cx43. An early phosphorylation of the nascent Cx43 takes place in the ER and Golgi apparatus and is probably important for the further processing steps, i.e. assembly of connexins and gap junction plaque formation [23]. Moreover, Musil and Goodenough [20] also showed that phosphorylation of the nascent Cx43 to the 46-kDa species (Cx43-P<sub>2</sub>) occurs soon after arrival on the cell surface and that phosphorylation of connexin hemichannels seems to be necessary for a normal gap junction function.

Already in the early 90s Musil and Goodenough [24, 25] found that oligomerization of the Cx43 protein into hexamers occurs after leaving the ER and in further studies it was demonstrated that this assembly into connexons is carried out in the *trans*-Golgi network during their passage through the Golgi apparatus [26, 27].

After oligomerization, two ways of delivering Cx43 to the plasma membrane have been described: to a minor extent Cx43 hemichannels (the connexons) seem to be directly inserted into plasma membrane, in contrast to the majority of Cx43 hemichannels, which follow the secretory pathway. After oligomerization of the connexins, the resulting connexon passes the cisternae of the Golgi apparatus and finally is transported within vesicles to the plasma membrane along microtubular filaments [28] (see also fig. 2).

The mechanism how connexons are integrated into the plasma membrane and how connexin hemichannels form a functional gap junction plaque is not yet fully understood. After delivery of the connexons to the plasma membrane insertion of connexons appear to occur without preselected localization, i.e. they are inserted over the entire nonjunctional plasma membrane surface, rather than with a direct assignment of certain connexons to already existing gap junction channels. The connexons enter the plasma membrane in small



**Fig. 2.** The scheme shows the life cycle of connexins including connexin synthesis, transfer, assembly and degradation pathways. In the lower left corner an annular gap junction is depicted. Adapted and modified from Sáez et al. [28].

groups and by lateral movement in the plane of the plasma membrane they may reach the outer rim of a connexon cluster. As a result, this accretion of newly arrived connexons with the outer rim of a gap junction cluster promotes growth of a gap junction plaque [29, 30].

After delivery of connexin hemichannels to the plasma membrane, the hemichannels of neighboring cells register and pair via interactions of their extracellular loops thereby forming a complete dodecameric gap junction channel.

This process seems to be facilitated by calcium-dependent cell adhesion molecules such as L-CAM (E-cadherin) [31]. Other cadherins such as A-CAM (N-cadherin) have also been described to be involved in gap junction formation [32]. In experiments using siRNA knockdown, Wei et al. [33] could show using NIH3T3 cells that cell surface expression of Cx43 requires N-cadherin and they suggested that N-cadherin and N-cadherin-associated proteins (catenins) play a dominant role in gap junction formation. But the role of cadherins seems to be complex and cell type specific: in 1997, Wang and Rose [34] demonstrated that cadherin-expressing mouse L cells express less Cx43 gap junction proteins than cadherin-lacking mouse L cells after stimulation with forskolin.

Besides the cadherins also  $\alpha$ -catenin, a cadherin-associated intracellular protein, which is considered to be pivotal for the adhesive function of cadherin molecules seems to play a role in gap junction formation. In 1997, Fujimoto et al. [35] demonstrated colocalization of E-cadherin and  $\alpha$ -catenin with Cx26 and Cx32 in regenerating hepatocytes and suggested that a cadherin-catenin complex may act as a focus for gap junction plaque formation. In rat cardiomyocytes, Wu et al. [36] could show an association between Cx43,  $\alpha$ - and  $\beta$ -catenin and ZO-1 (zonula occludens protein) and they demonstrated that this association is required for the development of gap junction formation. However, further research in this field has to be done to evaluate the role of cell adhesion molecules.

Furthermore a binding domain for the second PDZ domain of ZO-1 – a membrane-associated guanylate kinase (MAGUK) – has been found in the Cx43 C-terminal tail and it has been demonstrated that this Cx43/ZO-1 complex is linked to actin filaments via  $\alpha$ -spectrin [37–39]. It has been proposed that this interaction could possibly be a key event in localizing Cx43 gap junction proteins to the intercalated disk of cardiomyocytes and that this could be a mechanism for targeting connexins to specific regions in polarized cells [40]. Nevertheless, tagging of the C-terminus of Cx43 although abolishing ZO-1 interaction does not prevent gap junction formation [41]. A recent study by Hunter et al. [42] with wild-type Cx43 transfected HeLa cells revealed that in these cells wild-type Cx43 forms small punctate gap junctions. In the same study, in another set of experiments, the Cx43 C-terminus tail was tagged with green fluorescent protein (GFP) and it could be demonstrated that, although GFP prevented ZO-1 binding, gap junction plaques were formed. Surprisingly, gap junction morphology was different since the Cx43-GFP formed larger and more broadly distributed gap junction plaques compared with wild-type Cx43. These observations led to the hypothesis that ZO-1 might be involved in gap junction turnover rather than in localizing connexons to certain membrane regions. Consistent with this hypothesis, Barker et al. [43] found that in intact myocardium association between Cx43 and ZO-1 is only moderate but association



and colocalization increases after disruption of cardiomyocytes by collagenase treatment, a treatment which is known to break up intercellular couplings and lead to formation of cytoplasmic gap junctional vesicles known as annular junctions [44]. These annular gap junctions are internalization products of gap junction plaque fragments generated by endocytosis of both junctional membranes into the cytoplasm of one of the neighboring cells for further degradation. Cytoskeletal elements are involved in this degradation process as could be demonstrated by Murray et al. [45], who showed that disruption of microfilaments resulted in an increase in annular gap junction size and a decrease in annular gap junction number. They suggested that myosin-containing cytoskeletal elements might play a role in annular gap junction turnover.

### **Connexin Degradation**

The half-life of connexins, untypical of a structural membrane protein, is rather short, ranging from 1 to 5 h [46, 47]. However, some connexins seem to be more stable, as could be demonstrated for lens fiber Cx45 and Cx56, which exhibited a half-life of about 90 h in vitro [48].

In degradation studies of gap junction channels, several authors came to the conclusion that once a complete dodecameric gap junction channel has been formed it cannot be divided into hemichannels (connexons) again. Instead, one of the neighboring cell internalizes the entire gap junction channel. Electron microscopic studies revealed the existence of vesicular double-membrane gap-junction-like structures within the cytoplasm of cells, which are called ‘annular gap junctions’ and are the product of an invagination of both junctional membranes into the cytoplasm of one of the two contacting cells [49–52]. Which of the two cells receives the removed gap junctions remains unclear. However, internalization of entire gap junctions does not seem to be the only mechanism to regulate gap junction turnover since annular gap junctions are not found in all tissue types: in liver cells, for example, annular gap junctions are very rare or almost absent. In their study using regenerating mouse liver cells, Fujimoto et al. [35] proposed that gap junction plaques are removed from cell surface by a disassembly of whole gap junction channels into connexons, though they had no proof of how these disaggregated connexons or connexins are further degraded. They suggested that the connexons are internalized within endocytotic vesicles and are degraded within lysosomes. Recent observations of Lauf et al. [30] and Gaietta et al. [53] came to a similar assumption: these groups found that newly synthesized Cx43 is accrued along the outer rim of a gap junction plaque, whereas older channels are released from the plaque center. Thus, besides the mechanism of invagination of both junctional membranes into one

cell, an additional process must exist that continuously removes older channels from gap junction plaques, although such a mechanism has not yet been proven.

In pulse-chase experiments using isolated perfused rat hearts Beardslee et al. [54] calculated a half-life of the main cardiac connexin Cx43 of 1.3 h. The authors could also demonstrate *in vivo* that inhibition of either the proteasomal or the lysosomal pathway resulted in a significant increase in Cx43. Interestingly, inhibition of proteasomal Cx43 degradation resulted in an increase in nonphosphorylated Cx43 (but see below) whereas by inhibition of lysosomal proteolysis phosphorylated isoforms of Cx43 accumulated in the hearts. Since proteasomal proteolysis is the major pathway to degrade nuclear and cytosolic proteins, it is reasonable to assume that misfolded (nonphosphorylated) connexins trafficking through the ER/Golgi apparatus are degraded in the proteasome, which thereby serves as a quality control, whereas connexins assembled at the cell surface to functional intact gap junction channels (most of them are phosphorylated) are digested within lysosomes after removal from the cell membrane. These observations are consistent with *in vivo* experiments of VanSlyke and Musil [55], which demonstrated for the first time that misfolded connexins can be dislocated from the ER in an intact form to undergo degradation. The dislocation of connexins from the ER is an ATP-consuming process and the authors assumed that 26S proteasomes together with cap ATPases bind to the ER to extract connexins for degradation. Mild oxidative or thermal stress resulted in a marked reduction of connexin dislocation from the ER and in a significant increase in gap junction formation and function. This process could be interpreted as an adaptation to cell injury. In diseases such as myocardial infarction or stroke, the upregulation of gap junction formation in the border zone of the infarction might be considered as a response to the ischemia-reperfusion injury and as an attempt to restore cell function of impaired but not irreversibly damaged cells. Since it is well known that gap junction channels provide not only electrical but also metabolic coupling of cells, a transfer of antioxidative molecules such as glutathione from the normal unaffected tissue to the infarction border zone might occur and thereby enhance cell survival [56].

The amount of proteasomal versus lysosomal degradation of connexins may be cell type specific and has not been defined exactly yet. Furthermore, it should be pointed out that connexin degradation may follow different pathways in communication-competent cells (e.g. cardiomyocytes) or communication-deficient cells as, for example, tumor cells.

In a study on cells with inefficient gap junction assembly, Musil et al. [57] demonstrated that inhibition of proteasomal degradation of Cx43 was associated with an increase in Cx43 phosphorylation, gap junction formation and intercellular dye coupling. The effect of proteasomal blockers on gap junction assembly was mimicked by treatment of an assembly-inefficient cell line with

inhibitors of protein synthesis (cycloheximide), and Musil et al. proposed that an additional not yet identified short-lived protein may be required for proteasomal connexin degradation. This protein might either be involved in polyubiquitination of connexins or of other proteins which facilitate targeting connexins to proteasomal degradation or it might be involved in unfolding connexins for insertion into the catalytic core of proteasomes. Moreover, degradation of mutant Cx43 was sensitive to proteasomal blockers but was insensitive to inhibition of protein synthesis with cycloheximide. These experimental results suggested that proteasomal degradation of native but not of misfolded connexins involved a cycloheximide-sensitive step. The putative mediator addressed in this study has not been identified yet. In contrast, inhibitors of the lysosomal pathway (chloroquine or leupeptin) slowed down Cx43 degradation but were unable to upregulate gap junction assembly, consistent with the fact that these inhibitors, although preventing proteolysis within the lysosomal system, do not interfere with transport of proteins to the lysosomes. As a consequence, Cx43 accumulated in cytosolic vesicles. Since not only Cx43, but also the structurally quite different Cx32, showed comparable experimental results, the authors concluded that their model of connexin degradation may also be applied to additional members of the connexin family.

In a recent study, Qin et al. [58], using two cell lines (communication-competent and communication-deficient cells) reported on a new model in which lysosomes play a key role in degrading Cx43. Based on their results with lysosomal inhibitors, they suggested that lysosomes were not only involved in the breakdown of annular gap junctions but also in degradation of newly synthesized Cx43. They proposed a by-pass mechanism which directly targets newly synthesized connexins to the lysosomes without first being transported and integrated in the plasma membrane. In contrast, inhibition of the proteasomal pathway did not alter the total amount of steady-state levels of Cx43 in their study but, consistent with Musil et al. [57], resulted in an increase in Cx43 phosphorylation (the difference to Beardslee et al. [54] might be explained by the use of another cell line/species) and in the appearance of oversized gap junction plaques. Thus, the authors concluded that proteasomes are indirectly involved in the regulation of life cycle and turnover of Cx43 since they regulate the maturation or stability of gap junctions, whereas the lysosome is the main target for gap junction degradation. This thesis is supported by a recent study by Thomas et al. [59] in which the authors suggested that a tyrosine-based sorting signal in the C-terminus of Cx43 gives the go-ahead for Cx43 internalization and degradation in the lysosomal compartment.

It is widely accepted that polyubiquitination of proteins is a signal for the proteolytic pathway to recognize proteins marked for degradation [60]. Thus, another point to address is the question whether ubiquitination of connexins

occurs. Indeed, in contrast to Qin et al. [58], Laing and Beyer [61], demonstrated using Chinese hamster ovary cells that polyubiquitination of Cx43 does occur; their data suggested that the proteasomal pathway might be the major mechanism for Cx43 degradation and led them to conclude that lysosomes play but a minor role in Cx43 proteolysis. Cx43 ubiquitination has also been found in rat liver epithelial cells [62]. Using the phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA), an accelerated protein-kinase-C- and partly mitogen-activated-protein-kinase (MAPK)-dependent monoubiquitination and concomitantly Cx43 hyperphosphorylation occurred. After ubiquitination, Cx43 was internalized and degraded involving the lysosomal pathway.

However, the exact molecular mechanisms of connexin ubiquitination, internalization and degradation are currently unknown. Thus, further experimental studies are required to unravel the life cycle of the connexins.

In the past years, important insights into the life cycle of gap junctions have been achieved showing a highly dynamic regulation/turnover of number and localization of gap junctions as an important step defining the geometry and biophysics of a cellular network. However, there are still a number of open questions and much work will have to be done to allow integration of these mechanisms into the complex physiology and pathophysiology of the cardiovascular system.

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## Gap Junctions and Propagation of the Cardiac Action Potential

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### Abstract

Pacemaker cells in the heart generate periodic electrical signals that are conducted to the working myocardium via the specialized conduction system. Effective cell-to-cell communication is critical for rapid, uniform conduction of cardiac action potentials – a prerequisite for effective, synchronized cardiac contraction. Local circuit currents form the basis of the depolarization wave front in the working myocardium. These currents flow from cell to cell via gap junction channels. In this chapter, we trace the path of the action potential from its generation in the sinus node to propagation through the working myocardium, with a detailed discussion of the role of gap junctions. First, we review the transmembrane ionic currents and the basic principles of conduction of the action potential to the working myocardium via the specialized tissues of the heart. Next, we consider the relative contribution of cell geometry, size, and gap junction conductance. These factors are examined in terms of their source-to-sink relationships. Lastly, we will discuss new insights into the importance of gap junctions in cardiac conduction in health and disease which have been gained from high resolution optical mapping in connexin-deficient mice.

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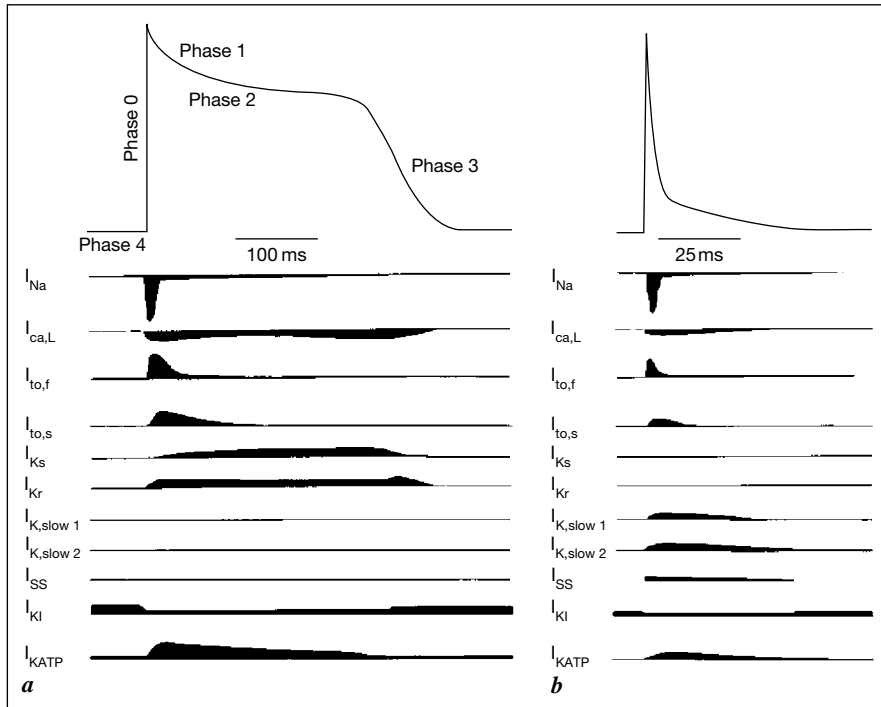
### Mechanisms of Action Potential Generation

Action potentials are regenerative signals produced by pacemaker cells, specialized conducting tissue within the heart, and by working myocytes. In 1924, Willem Einthoven was awarded the Nobel Prize in Medicine/Physiology for his accurate recordings of the human electrocardiogram (ECG) and the subsequent development of the ECG into a clinically useful tool. It was recognized early on that the surface ECG represented the electrical signal that precedes contraction of the heart muscle. By 1913, it was evident that rapid heart rates could be caused by



abnormal conduction of electrical signals in the heart, called re-entry [1]. The advent of more modern molecular and electrophysiological techniques has allowed for the study of the mechanisms responsible for generation and conduction of more complex signals [2–6]. Draper and Weidmann [7] were the first to record the resting membrane potential in cardiac cells using microelectrodes in 1951. A few years later, Weidmann [8] also predicted that low-resistance junctions between cells were important for propagation of the cardiac action potential. The development of single-cell electrode patch clamping made it possible to relate the generation of the cellular action potential to the summation of multiple-membrane ion channel currents [9]. The discovery of inward calcium current was also an important step in understanding signal propagation and electromechanical coupling in the myocardium [10]. In the last two to three decades, much work has been done to further characterize a variety of membrane ion channels along with the relationship between gene expression, structure and function. It is now possible to have a detailed understanding of the effects of antiarrhythmic drugs and genetic mutations on ion channel function. Indeed, a general appreciation of the generation of the action potential in a single cardiac myocyte is necessary for understanding intercellular propagation throughout the heart.

Changes in membrane potential ( $V_m$ ) are caused by alterations in membrane capacitance ( $C_m$ ) due to voltage-, time-, and concentration-dependent movement of ions across the cell membrane. Sodium, potassium, calcium and chloride ions move across the membrane via energy-dependent pumps, exchangers, and voltage-gated channels. The sum total of the ionic currents are given as  $I_{ion}$  in the following formula:  $dV_m/dt = -1/(C_m \cdot I_{ion})$ . By convention, the inward movement of positive ions results in a net positive  $dV_m/dt$  which depolarizes the cell. Figure 1 shows the major membrane ionic currents for the mouse (right) and human (left), and the resultant action potential. Rapid depolarization occurs as the fast inward sodium current generates the initial peak of the action potential, once minimum membrane depolarization threshold is reached. When the inward voltage upstroke reaches approximately  $-40$  mV, the L-type calcium channels are activated ( $I_{CaL}$ ). The inward calcium current serves to maintain the plateau of the action potential against the outward delayed rectifying currents ( $I_{kr}$  and  $I_{ks}$ ) and activates calcium-activated calcium channels on the sarcoplasmic reticulum. The calcium channels slowly deactivate and the delayed rectifier potassium current begins to repolarize the cell. Early in the action potential, the  $Na^+/Ca^{2+}$  exchanger acts to extrude  $Na^+$  and later removes  $Ca^{2+}$  from the cell. During the late repolarization phase of the action potential, the inward rectifying potassium current,  $I_{K1}$  also contributes outward current which returns the potential to its normal resting value. It is evident from this figure that major differences in repolarizing currents are present between human and mouse cardiac cells. In human cells,  $I_{to}$  currents are responsible for phase 1 early repolarization while in the mouse this current serves as the



**Fig. 1.** Cardiac myocyte action potentials in humans (**a**) and mice (**b**), and the individual membrane ion channel currents that contribute to the action potential. Mice lack the inward potassium-rectifying currents,  $I_{Ks}$  and  $I_{Kr}$ , lack the plateau during phase 2 depolarization, and have shorter action potential duration. Reprinted with permission from Nerbonne [11].

major repolarizing current. In fact, species-dependent differences in the magnitude of ionic currents have been reported [11, 12].

### Conduction of Action Potentials

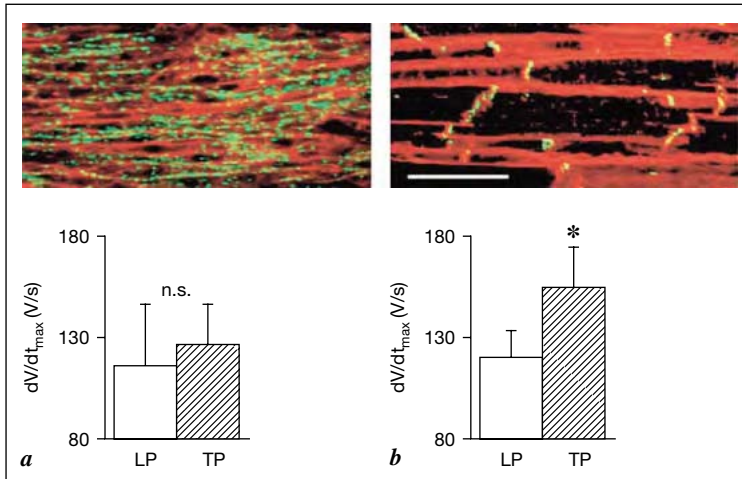
Once an action potential is generated, propagation requires longitudinal flow of electrical currents through the spaces between adjacent cells and across the plasma membranes of individual myocytes. In cardiac muscle cells, gap junctions provide large, nonselective channels that allow ions and other small molecules to diffuse freely between cells. In the adult heart, gap junctions form large channels concentrated at the ends of cells in the intercalated disks where cells are in close apposition to each other. In fetal and neonatal hearts, gap junctions are more evenly distributed throughout the cell membrane [13, 14].

The gap junction channels are formed by connexin (Cx) proteins and are essential for normal propagation of cardiac impulses and electromechanical coupling. Three major types of Cx proteins form gap junction channels in the heart; however, they are not expressed uniformly in all cardiac myocytes [15–17]. Each of these proteins, Cx40, Cx43, and Cx45, forms channels with unique electrophysiological properties due to differences in conductance, permeability, and voltage dependence [18]. Cx40 is present predominantly in the cells of the atria and conduction system, while Cx43 is the key subtype expressed by the working myocytes of both the atria and ventricles [17, 19, 20]. Cx45 is primarily confined to the sinoatrial and atrioventricular nodes [21, 22]. Recently, Kreuzberg et al. [23] have provided evidence that Cx30.2 is also prominently expressed in the mouse sinoatrial and atrioventricular nodes. In the same study they also demonstrated that Cx30.2 could form functional channels with other cardiac connexins suggesting that this connexin isoform may play a role in impulse initiation and propagation in the nodal regions of the heart.

Multiple electrophysiologic and morphologic parameters determine the characteristics of signal propagation in the heart. One important electrophysiologic parameter is excitability, which is dependent on the density and properties of the membrane ion channels. In addition, curvature of the wavefront influences conduction velocity. As the curvature of the wavefront increases (i.e., becomes more convex) the velocity slows, until the curvature reaches a critical value when propagation will fail completely [24–27]. Morphologic factors include the size and shape of the myocytes, distribution of nonexcitable tissues (fibrocytes, collagen, veins, arteries, nerves), presence of specialized conduction tissue (sinoatrial and atrioventricular nodes, His Bundle, Purkinje fibers), and the macroscopic formations of myocytes into larger structures (ventricles, atria). Local circuits of electrotonic ion currents are the basis of propagation of the depolarization wave front. These currents are generated by the inward flow of sodium ions and flow from cell to cell via the gap junction channels. The rapidity of these currents depends on the degree to which one cell contributes to the local current density in relation to its neighboring cells. Thus, effective cell-to-cell communication is critical for rapid, uniform conduction of the action potential – a prerequisite for uniform, synchronized cardiac contraction.

### **Role of Cell Size and Gap Junction Conductance in Propagation**

Pathologic changes in gap junction distribution can be an important factor in the development of cardiac arrhythmias. Alterations in the arrangement and quantity of gap junctions have been demonstrated during normal hypertrophy



**Fig. 2.** Experimental results using neonatal (**a**) and adult (**b**) intact myocytes. In the upper panels, cardiac gap junctions have been labeled with antibody to Cx43 and cell membranes were labeled with wheat germ agglutinin. The white bar represents 50  $\mu\text{m}$ . There is no significant difference in longitudinal (LP) and transverse (TP) conduction in the neonatal myocytes, while the adult myocytes demonstrate anisotropic conduction properties. Modified with permission, from Spach et al. [32].

and growth of myocytes, aging, and disease states including congestive heart failure and myocardial ischemia [28–30].

Joyner [31] provided the first detailed description of the relationship between cell size, cell coupling by gap junctions, and successful propagation. In this theoretical model, the speed of propagation depended on the magnitude of the intercellular resistance and the length of the cell (low-resistance segments).

Subsequently, in 2000, Spach et al. [32] further defined this relationship in experimental and computational models of propagation. They hypothesized that anisotropic growth effects are due to changes in scale (cell size) and cell-to-cell coupling by gap junctions. Using experimental data of cell geometry (size and shape), gap junction distribution, and action potential upstrokes, they simulated propagation in networks of adult canine and neonatal rat myocytes. Normal adult canine myocytes feature a large cell size with gap junctions preferentially distributed at the longitudinal ends of cells, in the intercalated disks. Neonatal rat myocytes are much smaller cells with gap junctions more evenly distributed throughout the cell membrane. These populations are represented in figure 2. Two virtual cell networks were also created. One network featured the large cell size of the canine myocytes combined with the uniform gap junction distribution of the neonatal myocytes. The second network consisted of small cells with

gap junctions predominantly localized at the ends of the cells. This series of simulations was designed to study the separate effects of cell geometry (size) and distribution of gap junctions on signal propagation. In this model, changes in cell size were found to be more important for action potential propagation than the distribution of gap junction channels. For example, a change from the adult-like gap junction distribution to the neonate-like pattern produces relatively little change in conduction properties. With either an adult or neonate-like pattern of gap junction distribution, changing the cell size resulted in large changes in conduction properties. These findings suggested two principles: first, if the degree of cell-to-cell coupling (quantity of gap junctions per unit area of cell membrane) decreases in relation to cell size, conduction will become more discontinuous. Secondly, for a similar cell size, increasing the number of gap junctions along the sides or ends of the cell will result in less discontinuous propagation.

### **Cell-to-Cell Coupling and Source-to-Sink Relationships**

Cell-to-cell coupling is directly related to the size, distribution, and specific properties of connexin proteins throughout the myocardium. Computational models have been formulated to explore the effect of these factors on cell-to-cell propagation of the action potential [32]. The simplest models consist of a linear arrangement of excitable cells. Using this model, the propagation of action potentials can be described in terms of a source-to-sink relationship. An excited cell behaves as a source of electrical charge (ions) to its neighboring cell, which in turn acts as an electrical load, or sink. The excited cell must provide sufficient electrical charge to bring the neighboring cell to depolarization threshold. When threshold is reached, the neighboring cell depolarizes and becomes the source of electrical charge for the next cell, continuing the propagation through the line of interconnected cells via local electrotonic currents. Mathematically, the source-to-sink relationship can be described in terms of a safety factor (SF) which defines the success of signal propagation [33]. The SF is the ratio of charge (time integral of net ionic currents) generated to the charge consumed by a single cell during the excitation cycle, expressed as  $SF = (Q_c + Q_{out})/Q_{in}$  when  $Q_m = 0$ . In this formula,  $Q_{in}$  represents the time integral of the total ionic currents moving into the cell and  $Q_{out}$  is equal to the time integral of the total ionic currents moving out of the cell. The third variable,  $Q_c$ , is the charge (capacitance) stored in the membrane as a result of depolarization, energy which will be contributed to the neighboring cells during repolarization.  $Q_m$  is the net charge of the cell, representing the amount of ionic charge generated over the duration of the action potential by the component ionic currents. When net charge is positive, the cell has consumed more charge

than it has produced and acts as a current sink. When the net charge is negative, the membrane is effectively a current source for the fiber. Near the peak of the action potential,  $Q_m$  returns to zero and the membrane has produced an amount of charge equal to that required to reach threshold and depolarize. This is an appropriate point in time to analyze the margin of safety for propagation because the numerator and denominator should, theoretically, be numerically equal. Thus, using this formulation, a  $SF > 1$  indicates propagation will be successful, while  $SF < 1$  indicates conduction failure. The margin of safety for propagation is indicated by the amount that  $SF$  is greater than 1.

The voltage change decrements exponentially with distance. In fact, only a fraction of the inward current of the excited cell(s) is used to depolarize the immediate neighboring cell(s). A good portion of the current is used to depolarize downstream cells. Though the cells far downstream are not depolarized above threshold, they represent a significant load on the depolarizing cell. This load draws charge away from the immediate capacitor (the discharging cell), and acts as a current sink. If this mode of propagation is viewed from a microscopic perspective, discontinuous cell-to-cell propagation occurs in a local all-or-none fashion as the impulse moves downstream. Each cardiac cell is connected to its neighbors through gap junction channels with resistance,  $R_j$ , significantly higher than the intracellular resistance,  $R_i$ . From a macroscopic perspective, the action potential appears to move at a uniform and continuous velocity through the myocardium.

The importance of gap junction resistance has become evident in several experiments [34–38]. In one elegant series of experiments, Fast and Kleber [34] set out to characterize conduction in linear arrays and sheets of culture myocytes. Using voltage-sensitive fluorescent dyes, conduction properties were related to cellular architecture, presence of discontinuities (nonconducting cells), and gap junction distribution. Intracytoplasmic conduction times averaged  $38 \mu s$ , for a typical myocyte  $30 \mu m$  long. However, cell-to-cell conduction times through the same distance across an end-to-end connection increased to approximately  $118 \mu s$ . This conduction time can be decreased by placing multiple strands of myocytes together to form wider strands, or narrow sheets. In this case, the intra-cytoplasmic conduction time is increased to  $60 \mu s$  while the cell-to-cell conduction time is decreased to  $88 \mu s$ . Macroscopic conduction velocities were virtually identical for both linear single strands and multiple strands of cells ( $34$  and  $36$  cm/s, respectively). Lateral strands of cells may therefore provide an averaging effect, reducing the discontinuity imposed by the gap junction resistance. Mathematical models indicated that the averaging effect is caused by the convergence of local excitatory currents beyond the end-to-end connections combined with divergence of currents before the end-to-end connections. The current flow through lateral cellular connections effectively smoothes the excitation wavefront during longitudinal propagation.

It is possible that conditions that favor lateral uncoupling (e.g., fibrosis), will unmask these discontinuities and may play a pivotal role in the aged heart (see chapter contributed by S. Dhein, p. 198).

### **Propagation at Junctional Sites and Branch Points**

Several recent studies have greatly expanded our understanding of the role of macroscopic structures in wavefront propagation [24, 37, 39–41]. It has been known for some time that conduction velocity, and the safety factor for propagation are affected by gross anatomical geometry. Recently, we have learned that the size scale of alterations in myocardial geometry is similar to that which can induce wave breaks and lateral instabilities in myocardial tissue [24]. Anatomic boundaries and discontinuities, on the order of 1 mm or greater, can facilitate the development of unidirectional block setting the stage for re-entrant activity.

These effects can be explained by abrupt changes in wave front curvature and the source-to-sink relationship of excitatory current. High wave front curvature and a mismatch of source-to-sink excitatory current can be found at sharp-edge obstacles, such as a scar resulting from a myocardial infarction, acute-angled branch points, such as a branching His-Purkinje fiber and at Purkinje muscle junctions. The source-to-sink mismatch occurring at these branch points can result in nonuniform anisotropic conduction and the development of functional unidirectional block.

Interestingly, we have recently described the first in vivo demonstration that lowering electrical coupling between cells can facilitate conduction under certain conditions. In genetically engineered mice with profound deficiency in Cx43, highly abnormal ventricular activation patterns are seen during normal sinus rhythm [36]. As a result of poor cell-to-cell coupling in the working myocardium, successful conduction across normally quiescent Purkinje-ventricular myocyte junctions was observed. This greatly increased the number of epicardial breakthrough activation sites. The resulting activation profiles are associated with nonuniform anisotropic conduction and an increase in wave front collisions, which can initiate re-entrant arrhythmias. We speculate that the decrease in electrical coupling during the early stages of myocardial ischemia may alter sinus activation patterns through a similar mechanism and may be an important contributing factor in the development of ischemia-induced arrhythmias.

### **Continuous versus Discontinuous Conduction**

There are two explanations for the nature of the apparent discontinuous conduction between myocytes. The current-to-load mismatch may represent

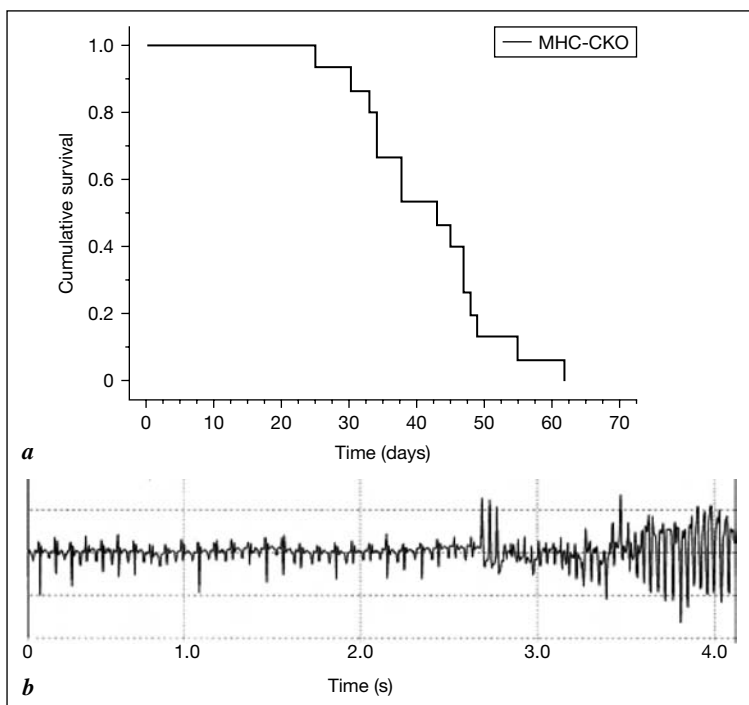
either differences in charge capacities of the individual cells or in the electrical coupling between the cells, or both. Several studies have demonstrated that strong cell-to-cell coupling is necessary for effective propagation of the action potential [35, 42, 43]. In sheets of myocardial cells, small intercellular clefts (less than one cell length) have minimal effect on propagation. However, larger clefts (150  $\mu\text{m}$  or larger) result in discontinuous conduction and block [39]. Cultured myocardial cells treated with palmitoleic acid, a gap junction inhibitor, do not have normal activation patterns. Very slow conduction occurs and typically takes a more meandering path, due to the heterogeneous inactivation of gap junctions. However, palmitoleic acid is a nonspecific inhibitor of ion channels and likely affects nonjunctional ion channels such as K, Na, and Ca as well. For this reason, connexin-deficient mouse models have been developed to explore the effect of poor cell-to-cell coupling in vivo and in vitro [40, 42, 44–48].

### **High-Resolution Mapping of Cardiac Excitation in Mice**

Alterations in Cx43 levels have been shown to cause slowed conduction and lead to the formation of lethal arrhythmias in mice [42]. Human heart disease is associated with two types of connexin remodeling: (1) structural remodeling and (2) remodeling of protein expression levels. Structural remodeling occurs when the normal cellular arrangement and organization of connexin proteins is disrupted. Studies involving failing hearts taken from patients during cardiac transplantation have shown that the normal, ordered distribution of Cx43 is lost in the areas bordering infarcted myocardium [30]. Significant reductions in the expression of Cx43 protein have been demonstrated by quantitative Northern and Western blotting techniques in human diseased hearts. It is possible that these two remodeling processes lead to distinctly different electrophysiological phenotypes. The topic of connexin remodeling is extensively covered in the chapter contributed by N.J. Severs (p. 228).

The use of genetically modified animals to define Cx43 function has, until recently, been somewhat limited. Unrestricted deletion of Cx43 results in a perinatal lethal phenotype characterized by a severe right ventricular outflow tract obstruction. Studies in these animals have consequently been restricted to the prenatal period [43]. Recently, techniques of optical epicardial mapping in whole-heart preparations have been applied to adult Cx43-deficient murine models. Morley et al. [49] examined epicardial conduction velocities in heterozygous Cx43-deficient animals and showed no difference in conduction velocities compared to control animals. We concluded that a single null allele

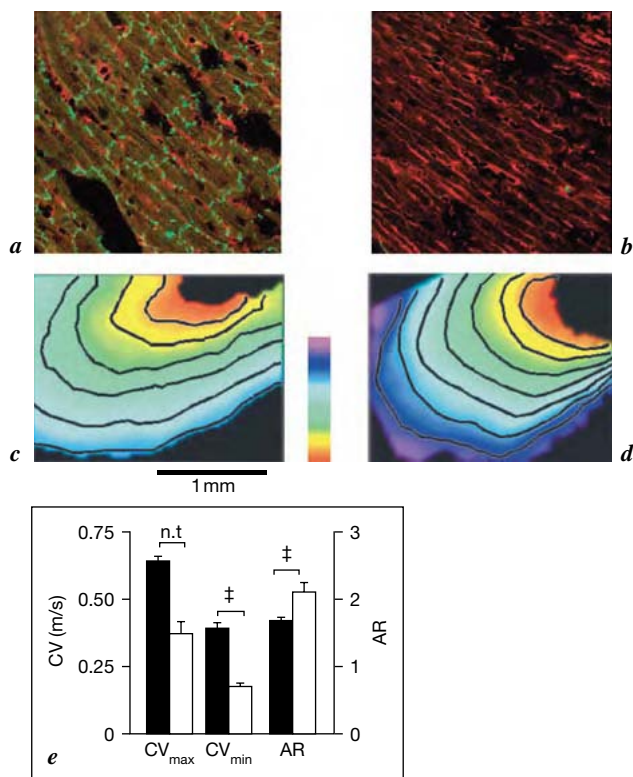




**Fig. 3.** *a* Kaplan-Meier survival curve for connexin43 MHC knockout mice. All MHC-CKO mice died by 3 months of age. There were no deaths in the control group. *b* Episodes of VF immediately preceding the deaths of 3 of the animals were captured on telemetric recordings. Modified with permission, from Gutstein et al. [42].

for Cx43 resulted in little or no alteration in epicardial conduction properties. This is consistent with theoretical predictions, based on computational models of cell-to-cell coupling. Early computer simulations demonstrated that gap junctional resistance can be increased by a factor of ten before significant effects on conduction properties are noted [50]. Despite these findings, Cx43 heterozygous hearts subjected to acute regional ischemia have a markedly higher incidence of sustained ventricular arrhythmias compared to control animals [51].

More recent studies have involved conditional, cardiac-specific Cx43 knockout animals where Cx43 is nearly absent in the ventricular myocardium [42]. Generating mice with cardiac-restricted knockout of Cx43 avoids the problem of perinatal lethality seen in homozygous Cx43 null animals. These studies have shown highly significant conduction slowing and sudden arrhythmic death by 3 months of age (figures 3 and 4) [42]. Successful impulse propagation is



**Fig. 4.** Immunofluorescent staining for Cx43 in wild-type (*a*) and MHC-CKO hearts (*b*). Hearts were stained for Cx43, counter-stained with wheat-germ agglutinin, and imaged by confocal microscopy. Normal distribution of gap junction is seen in the wild type, with nearly absent Cx43 in the knockout animals. *c, d* Activation maps of wild-type (*c*) and Cx43 knockout animals (*d*) during epicardial pacing. Maximum conduction velocity in this wild-type heart is 0.58 m/s compared to 0.42 m/s in the CKO mouse. *e* Average conduction velocities and anisotropic ratio derived from optical mapping studies. Maximum and minimum conduction velocities are up to 50% slower in Cx43 knockout animals (white bars) compared to wild-type controls (black bars). Modified with permission, from Gutstein et al. [42].

maintained, despite slowed conduction velocity, until malignant arrhythmias occur. This is consistent with a paradoxical increase in safety factor in the setting of reduced cell-to-cell coupling, as discussed previously. Thus, in the clinical setting of chronic heart disease plus an additional insult, such as acute ischemia, cell-to-cell uncoupling can result in alterations of gross electrophysiology and predispose to malignant cardiac arrhythmias.

## Conclusions and Future Studies

Sudden cardiac death (SCD) is defined as death from unexpected circulatory arrest, usually due to a cardiac arrhythmia occurring within 1 h of onset of symptoms. The occurrence in the United States is up to 400,000 cases annually [52]. Patients with existing heart disease, specifically depressed left ventricular systolic function and congestive heart failure, are at high risk for SCD. Despite aggressive pharmacologic therapy, these patients have an annual incidence of SCD up to 7% [53–55]. Anti-arrhythmic drug therapy has been associated with higher mortality rates, due to pro-arrhythmic effects. Currently, the most effective therapy for the prevention of SCD is implantable cardiac defibrillators. However, these devices require surgical implantation, are extremely expensive, and must be monitored frequently. Thus, new therapeutic targets to reduce the incidence of SCD in high-risk patients need to be found.

We have shown the importance in gap junctional coupling for normal signal propagation in normal and pathologic conditions. Coupling by gap junctions plays a vital role in modulating conduction in terms of the source-to-sink relationship. Remodeling of gap junction distribution and expression patterns have been shown to alter conduction velocity in animal studies and in hearts taken from patients with cardiomyopathy and have been associated with the development of lethal arrhythmias in homozygous Cx43 knockout animals. Animals with heterozygous knockout of Cx43 (+/– alleles) are observed to have minimal differences in conduction patterns compared to normal animals. However, these animals are predisposed to malignant arrhythmias with an additional insult, such as acute ischemia. Remodeling of gap junctions alone is not responsible for all of the electrophysiologic changes associated with clinical cardiac disease. However, it is clearly an important factor in the broad picture of left ventricle mechanical and electrical remodeling which predisposes patients to malignant arrhythmias and high mortality. Further studies in animals are needed to determine potential therapeutic targets of gap junction remodeling, such as connexin protein gene expression, connexin protein transport within the cell, gap junction assembly, or inappropriate transport of gap junctions to the lateral membranes. In the future, drug therapy targeting gap junction remodeling may offer significant reduction in mortality in high-risk patients at much lower cost than currently available therapies.

## Acknowledgements

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## Micropatterns of Propagation

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### Abstract

Alterations in microscopic conduction could contribute to microreentry and arrhythmogenesis in pathological settings. This chapter reviews microconduction in the ventricular myocardium. Gap junctions play a significant role in longitudinal and transverse propagation of the action potential wavefront in the ventricle. Studies of microscopic conduction in patterned cultures of neonatal rodent myocytes have provided novel insights into the role of gap junctions, the effects of uncoupling versus altered excitability, and the contribution of discontinuities and branching. Decreased gap junctional coupling can contribute to slowing of conduction and development of unidirectional block. However, in the setting of structural inhomogeneities and unbalanced current source and load, decreased coupling can, at times, improve conduction and be 'anti-arrhythmic,' attesting to the complexity of intercellular coupling as a therapeutic target. Genetically engineered mouse models of Cx43 depletion demonstrate slow conduction and arrhythmogenesis that appears to be reentrant in nature. Studies in these models provide novel insights into the contribution of gap junctions to impulse propagation and arrhythmogenesis in the intact heart. Overall, gap junction expression, distribution and heterogeneity are important contributors to microscopic conduction, and alterations in any of these can contribute to the development of an arrhythmogenic substrate in pathological states.

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In pathophysiologic settings, such as myocardial ischemia and myocardial infarction, sudden cardiac death is frequently due to reentrant arrhythmias that result from altered propagation of the action potential (AP) [1]. While macroreentry is common, microreentry could arise from abnormalities in microscopic conduction due to alterations in intercellular gap junctions. Understanding of micropatterns of conduction is important for the development of therapeutic approaches for the prevention and treatment of these lethal arrhythmias.

The aims of this chapter are to review microconduction in ventricular tissue and to consider the role of gap junctions in the process. We first introduce basic concepts in cellular electrophysiology, focusing on how the impulse spreads from

one cell to the next, how cellular coupling via gap junctions influences propagation of the AP on a microscopic level, and how perturbation in gap junctions and other factors that influence cell-to-cell interactions may result in arrhythmias. We then focus on studies of microscopic conduction obtained primarily from high-density optical recordings of cardiac activation in cardiac cells and tissue from control animals and from genetically engineered animals with deletions of the gap junctional protein connexin Cx43.

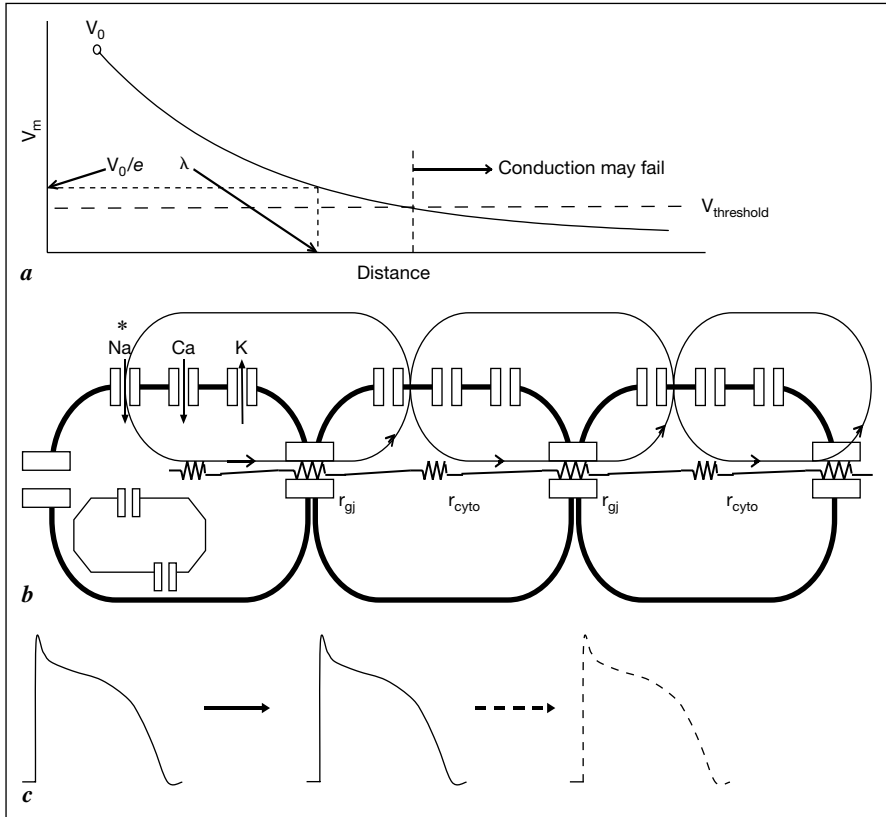
### Propagation of the Action Potential

Generation of the cardiac AP is a complex process in which there is coordinated spatial and temporal summation of ionic currents through multiple ion channels expressed in cardiac myocytes [for review, see ref. 2]. The individual ion currents that make up the cardiac AP will not be discussed here, but it is important to understand how membrane voltage ( $V_m$ ) is influenced by ionic currents in myocytes. In a single cell, temporal change in  $V_m$  is described by

$$\frac{dV_m}{dt} = -\frac{1}{C_m} I_{ion},$$

where  $C_m$  is the membrane capacity, and  $I_{ion}$  is the sum of ionic currents. The change in  $V_m$  is directly proportional to the ionic current through the channels. Note that inward movement of cations, such as  $Na^+$  current, results in positive  $dV_m/dt$ , thus depolarization, whereas outward  $K^+$  current repolarizes  $V_m$ . In the heart, however, where cells are coupled by gap junctions to neighboring cells, the relationship between  $V_m$  and  $I_{ion}$  is much more complex and non-linear. When a depolarizing current ('source') is generated, it is divided between charging the membrane nearby and depolarizing the adjacent cell (for the sake of simplicity, denoted 'sink') through gap junctions. The amount of current spent in depolarizing the downstream cell depends on the axial resistance, a part of which is gap junctional resistance. Conceptually, if there were a single source of current (asterisk in fig. 1b),  $V_m$  would gradually drop (fig. 1a) electrotonically along the fiber length. In the heart, if  $V_m$  is still depolarized enough beyond the threshold for the voltage-gated  $Na^+$  channel to open, the inward, depolarizing current flows at that site (above the  $V_{threshold}$ ), and renews (regenerates) the start of AP in the adjacent cell (fig. 1c, second cell). Conversely, if no regenerative currents occur along the fiber while  $V_m$  drops below the  $V_{threshold}$ , propagation of the AP fails (fig. 1c, third cell). This drop in  $V_m$  generally follows an exponential decay function and depends on membrane integrity (e.g. a leaky membrane causes  $V_m$  to decline faster), internal resistance (in this case a combination of  $r_{gj}$  and  $r_{cyto}$  in fig. 1) and, to a minor extent, external resistance. In a cable model of excitable





**Fig. 1.** Schematic drawing of myocytes in a linear chain. **a** Electrotonic, exponential drop in  $V_m$  from the initial  $V_0$ , where inward, depolarizing current has occurred. If no regenerative currents are produced (for example, by the  $Na^+$  channels in cell 2),  $V_m$  may decay beyond the threshold ( $V_{threshold}$ ) for  $Na^+$  channel activation, and conduction may fail. **b** Three cells are shown connected by gap junctions.  $I_{ion}$  is carried by various ion channels in the plasma membrane as well as sarcoplasmic reticulum (shown as elliptical structure within the cells). Cytoplasm and gap junctions have intrinsic resistance,  $r_{cyto}$  and  $r_{gj}$ , respectively, connected in series. Local circuit current, noted to progress counterclockwise, proceeds from right to left. **c** AP is generated in the first cell and propagates to the next cell, provided the electrotonic current is sufficient to depolarize its membrane beyond the  $V_{threshold}$  for the voltage-gated  $Na^+$  channel.

membrane, it can be characterized by a 'length constant' ( $\lambda$ ), which is the axial length at which  $V_m$  has dropped to  $1/e$  ( $\sim 1/2.7$ ) value of the  $V_0$  (fig. 1a). The length constant can be approximated by

$$\lambda = \sqrt{r_m / (r_i + r_o)},$$

where  $r_m$ ,  $r_i$ , and  $r_o$  are the membrane resistance, internal axial resistance (in fig. 1,  $r_i = r_{gi} + r_{cyto}$  since they are in series), and external resistance, respectively. In a setting where external resistance is relatively small as in typical experimental conditions where Purkinje fibers are perfused in a bath, the equation reduces to

$$\lambda = \sqrt{r_m / (r_{gi} + r_{cyto})}.$$

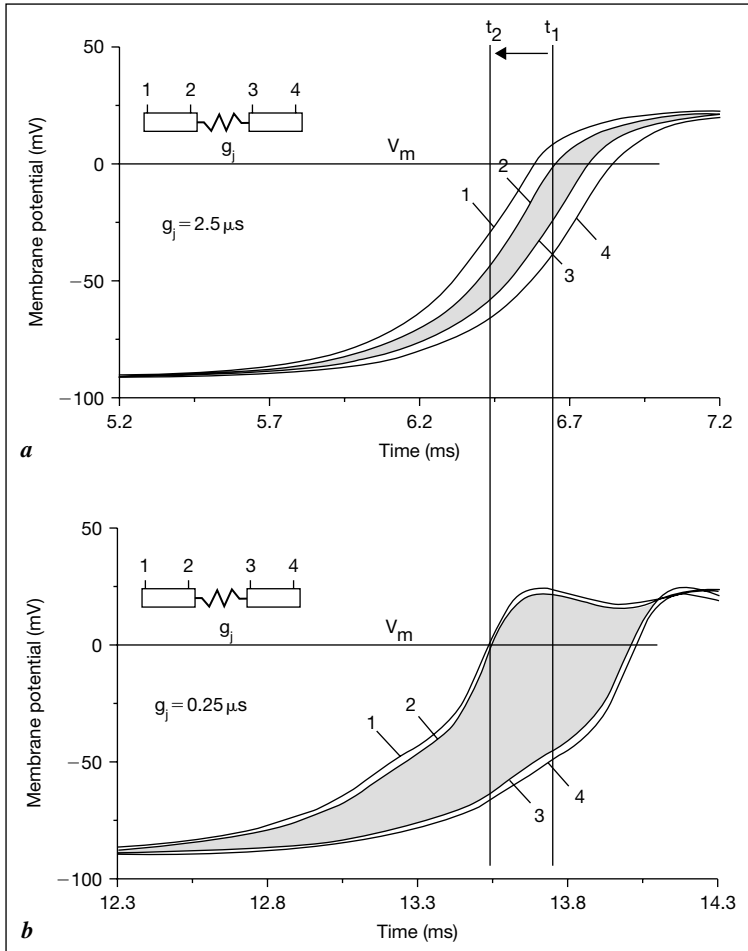
The length constant is a useful parameter in characterizing the cellular coupling because generally the larger it is, the faster is conduction. Therefore, gap junctions are an important determinant of conduction velocity.

As shown above,  $r_{cyto}$  and  $r_{gi}$  directly influence  $\lambda$  (the length constant of a linear strand of myocytes) and are influenced by gap junctional connections and the geometry of cells. Some studies have found a rough relationship between diameter and conduction velocity [3], but others have found conduction velocity to be quite constant regardless of the diameter, as in Purkinje fibers [4]. Such discrepancy indicates that there may be anisotropy within a fiber or that the extracellular space influences the conduction, suggesting that the ‘cell size’ and the extracellular milieu are also important factors in AP propagation.

The impact of reduced cellular coupling on the conduction velocity can be inferred from computer simulations of AP propagation in a continuous, linear strand of excitable cells. In figure 2 [5], AP upstrokes are dynamically simulated based on ion channel currents and gap junctional conductance, under a condition where external resistance is negligible. As shown in figure 2a, it takes almost as long to propagate an AP from point 2 to point 3 as it does from point 1 to point 2. Because the gap junctional clefts are much shorter than the lengths of myocytes that they join, conduction between the two cells that are coupled is saltatory with the conduction velocity through the gap junctional cleft between two cells being approximately 10% of that through the myoplasm. When coupling decreases (fig. 2b), propagation of AP through the gap junctional cleft slows significantly. Interestingly, conduction through the myoplasm (from 1 to 2 and from 3 to 4) speeds up. This phenomenon is partly responsible for the paradoxical increase in conduction when coupling is decreased in a certain manner.

## **Impulse Propagation through the Three-Dimensional Myocardium**

Propagation of impulse through the three-dimensional cardiac tissue is much more complex, and proceeds faster along the longitudinal direction (along the fiber length) than the transverse direction. This ‘anisotropy’ results from the



**Fig. 2.** Computer simulation of AP propagation in a linear chain of cells. Upstroke of APs at four sites in two coupled cells is shown. Shaded areas are time elapsed during propagation through the junction between the two cells (essentially via gap junctions). **a** When cell coupling is normal, duration of AP propagation through the length of the cell is similar to that through the cellular junction. **b** When gap junctions are uncoupled, transjunctional conduction time increases significantly. Interestingly, AP propagates faster through the length of the cell because of reduced current ‘sink’ (time lag between 1 and 2, or 3 and 4).  $V_m$  at 2 reaches 0 mV (an empiric level chosen for demonstration) faster in **b** than in **a** ( $t_1$  to  $t_2$ ). Modified from Shaw and Rudy [5] with permission.

intrinsic structure of the myocardial tissue. It can be a normal characteristic of tissues depending on the fiber axis and the distribution and functionality of gap junctions linking cells longitudinally and transversely. For example, the localization of gap junctions, particularly in adult tissues, is dominantly in end-to-end connections, leaving transverse electrical coupling with a smaller magnitude and less uniformity than longitudinal coupling, consistent with slower and more indirect propagation off the long axis [6–10]. It is the predominantly end-to-end distribution in tissues that favors anisotropic conduction, such as in Purkinje fibers and ventricular tissues, while the more diffuse distribution in the AV node would favor current distribution in all directions. Anisotropy can also be a result of disease, which alters the structural elements, such as fibrosis or hypertrophy, and provides the substrate for reentrant arrhythmias.

### Discontinuities

The heart had long been viewed as an electrically continuous medium (syncytium). However, as noted above, even in a linear structure of Purkinje fibers, extracellular milieu surrounding the fiber and anisotropy within the fiber influence AP propagation. In a three-dimensional tissue within which each myocyte is adjacent and may be coupled to  $>10$  other neighboring cells, more factors that are intrinsic and extrinsic to the myocytes impact on AP propagation and produce ‘discontinuous’ conduction on a microscopic scale.

To describe anisotropic propagation empirically on a macroscopic scale, Spach et al. [11, 12] proposed measurement of the effective axial resistivity,  $\overline{R}_a$ , which is the empiric value of internal resistivity to account for the observed speed of propagation along any direction, not just along the long axis of muscle fibers. Unlike  $r_i$  in the linear continuous cable theory, which incorporates only axial cytoplasmic resistivity ( $r_{\text{cyto}}$ /unit length) and end-to-end gap junctional resistivity ( $r_{\text{gj}}$ /unit length),  $\overline{R}_a$  also includes implicitly the influences of cellular geometry and packing, extracellular resistivities, side-to-side couplings, and other features. Equations based on axial resistivity have successfully predicted propagation on a macroscopic scale (several millimeters or more), even along pathways of a complex or heterogeneous structure. For example, excitation in young hearts spreads along smooth contours in directions off the long axis, indicating uniform anisotropy, but, in older preparations, the fast longitudinal path was narrow and had abrupt borders [13, 14]. Off the long axis, impulse propagated very slowly and in irregular or ‘zigzag’ fashion and often reached a transverse site at multiple different time points. This dissociation or fractionation indicates propagation by multiple paths, which would not occur in uniformly anisotropic tissue.

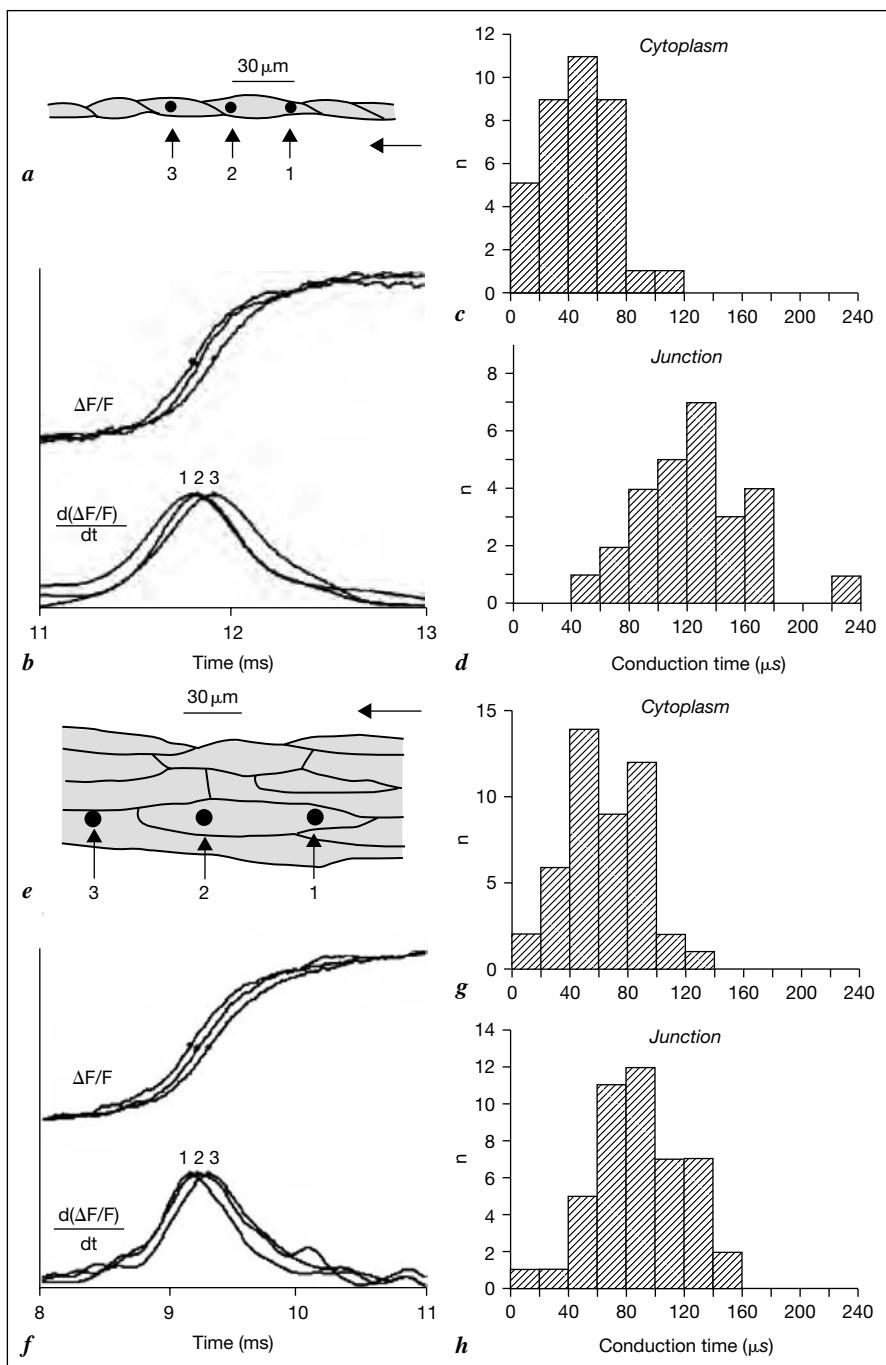
Spach et al. [12] proposed that microscopic discontinuities of axial resistance due to gap junctions underlie non-cable-like behavior of conduction and that these discontinuities were the basis for heterogeneous conduction and conduction block that led to arrhythmias. They assessed propagation by recording local activation time from the extracellular potential (with metal electrodes) and demonstrated the presence of local inhomogeneities of propagation (resolution  $<200\text{ }\mu\text{m}$ ). They demonstrated discontinuities of conduction in several types of cardiac tissues. In atrial and Purkinje tissue, discontinuities might arise from branching of trabeculae and longitudinal dissociation in cell strands [12]. In ventricular tissue, discontinuities in transverse propagation likely arise from connective tissue sheets and microvasculature that separate strands of ventricular myocytes [13, 15].

### Studies of Micropropagation in Patterned Cell Cultures

But there are questions as to whether extracellular potentials can adequately measure local excitation with sufficient spatial and temporal resolution (especially in tissue with nonuniform axial resistance) [16, 17] and whether transmembrane APs may provide more accurate measurement of local excitation. To address this, Fast and Kleber [18] have developed methods of optical recordings of APs (using voltage-sensitive dyes) in cultured neonatal rat ventricular myocytes in patterned cell monolayers.

In their initial study, they compared cytoplasmic and junctional propagation in one-dimensional cell chains (one cell wide) to that in two-dimensional cell strands (4–6 cells wide) [18]. They found that microscopic propagation was a discontinuous process with slowing at intercellular junctions. In one-dimensional cell chains, cytoplasmic conduction time averaged 38 ms while junctional conduction time averaged 118 ms (fig. 3a–d). The time difference between these two (80 ms) represents the average conduction delay introduced by a single end-to-end cell connection which corresponded to 51% of overall conduction time. In two-dimensional strands, junctional conduction time averaged 89 ms and cytoplasmic conduction time averaged 57 ms (fig. 3e–h). Thus

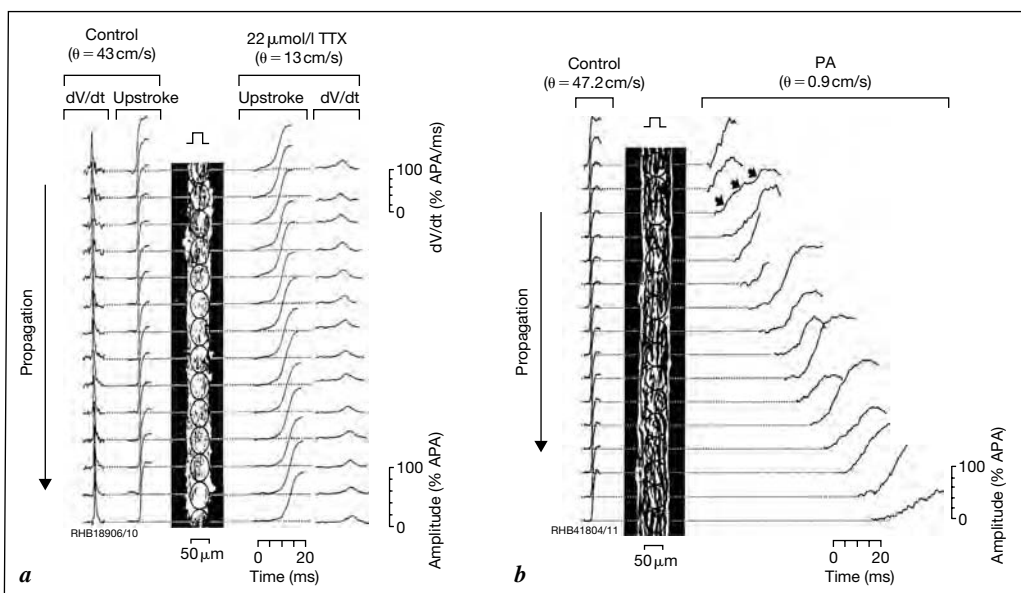
**Fig. 3.** Impulse propagation in one-dimensional cell chains (a–d) and two-dimensional cell strands (e–h). **a** Diagram of a portion of a cell chain (stained with the voltage-sensitive dye RH-237) with 1–3 denoting membrane areas sensed by three photodiodes. Arrow denotes direction of propagation. **b** Plot of potential-dependent changes in fluorescence and their time derivatives for the three photodiodes in **a**, with activation indicated by the points. **c, d** Histograms depicting cytoplasmic (**c, g**) and junctional (**d, h**) conduction times. **e–h** Similar set of diagrams (**e**), plots (**f**) and histograms (**g, h**) for a wide (4–6 cells in width) cell strand. From Fast and Kleber [18] with permission.



the gap junctional delay (of 32 ms) had decreased to 22% of overall conduction time, implying that lateral cell-to-cell connections decrease inhomogeneities (or smooth the discontinuities) in longitudinal conduction. In other words, inhomogeneities during longitudinal conduction are offset, at least in part, by current flow through lateral cell-to-cell connections. Their mathematical modeling further suggested that the averaging effect of lateral connections is due to lateral convergence of local excitatory current beyond and lateral divergence before end-to-end connections.

Fast and Kleber [19] went on to study the formation of unidirectional conduction block that is critical for reentrant excitation. Patterned cell cultures (cell growth in patterns similar to that in fig. 6a) were designed with a large central area with two types of channels – one narrow (width of 20–40  $\mu\text{m}$ ), the other wider (width of 100–140  $\mu\text{m}$ ). Transition regions (from narrow and wide strands) were optically imaged using a  $10 \times 10$  photodiode array (15- $\mu\text{m}$  resolution). They found that unidirectional conduction block, i.e. block of an impulse in an antegrade direction (from a strand to a larger area) but conduction in a retrograde direction, occurred only in narrow strands with an average width of 15  $\mu\text{m}$ ; but no conduction block was found in those with a width of 31  $\mu\text{m}$  or greater. Unidirectional conduction block arose from either geometric expansion alone (which led to directional differences in electrical load) or additional local depression of conduction. Impaired cell coupling was excluded by intact retrograde conduction. Mathematical modeling showed that decremental conduction was largely due to the electrotonus rather than by local ion currents. While this study found that in regions of abrupt expansion, a strand diameter of less than 15  $\mu\text{m}$  (1–2 cells) was a critical size below which unidirectional block developed, the critical size of strand diameter in adult heart tissue *in vivo* is likely to be larger because of differences in electrical coupling, surface-to-volume ratios of adult versus neonatal myocytes, and the three-dimensional structure of *in vivo* tissue [20].

Rohr et al. [21] showed that, in linear cell strands, conduction velocity (before block) is reduced to a far greater degree ( $\sim 98\%$ ) by partial gap junctional uncoupling with palmitoleic acid than by reduction of excitability ( $\sim 30\%$  with tetrodotoxin; fig. 4). These findings support the predictions from computer simulations that electrical uncoupling reduces conduction velocity to a greater degree than reduced  $I_{\text{Na}}$  [5], and suggest that electrical uncoupling results in an increased margin of safety of propagation. During partial uncoupling (fig. 5), activation wavefronts follow meandering pathways because of diversion around patches of completely uncoupled cells, resulting in slow conduction with ‘zig-zag’ activation that is reminiscent of that observed in infarcted myocardium [22]. High-resolution (10  $\mu\text{m}$ ) recordings in wide strands revealed stepwise activation indicating the presence of highly discontinuous conduction

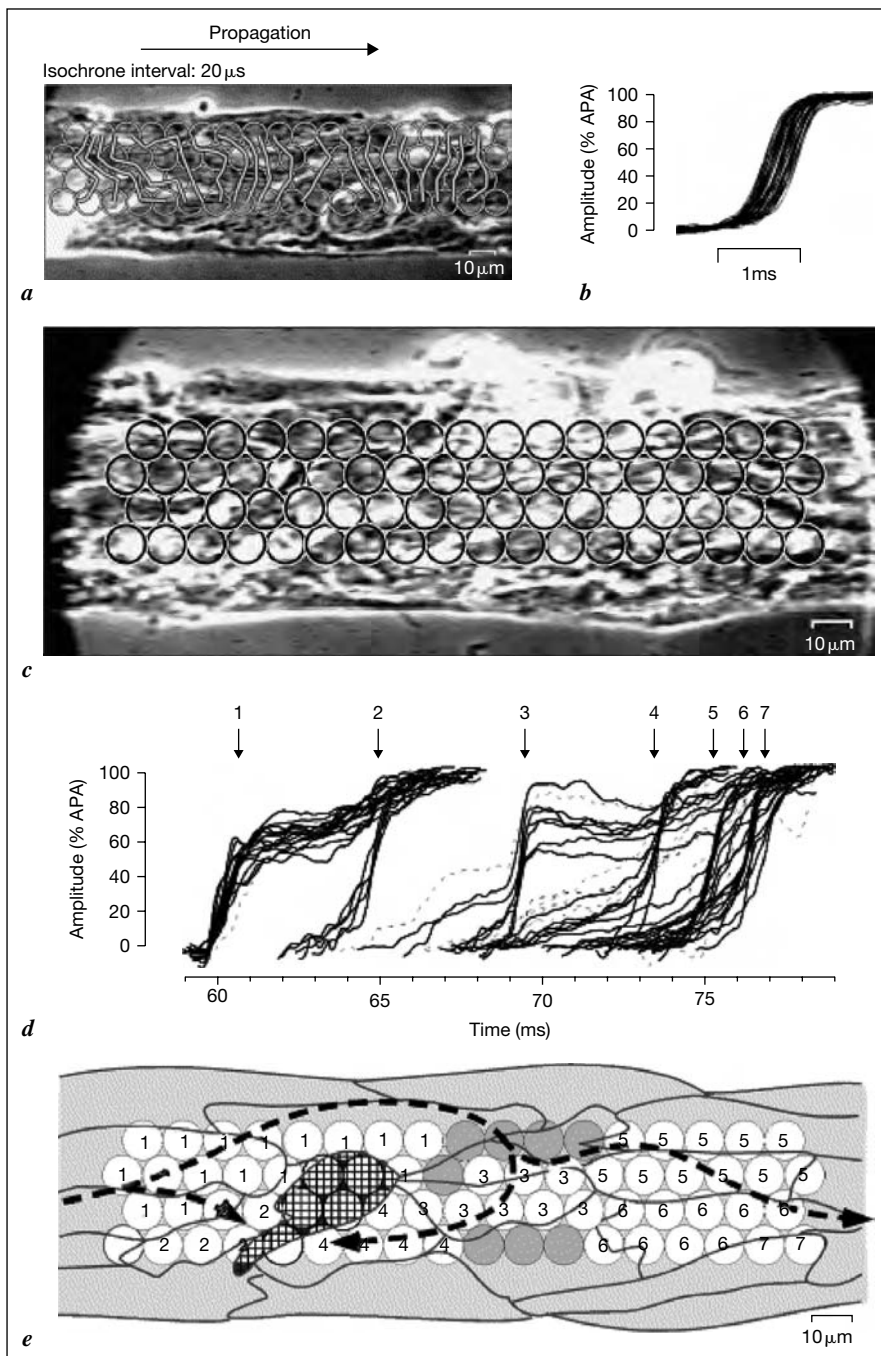


**Fig. 4.** Slowing of conduction by tetrodotoxin (TTX) and the gap junctional uncoupler palmitoleic acid (PA) in narrow cell strands. **a** The photograph in the middle shows the narrow cell strand preparation (stained with the voltage-sensitive dye di-4-ANEPPS) with the circles depicting individual photosensors. The arrow denotes the direction of propagation with 2-Hz stimulation. Shown are the tracings of voltage upstroke and  $dV/dt$  under control conditions (left) and with superfusion of TTX. **b** Conduction slowing with superfusion of PA and characteristics of microscopic impulse propagation during ultraslow conduction with gap junctional uncoupler are shown. Similar set of diagrams with strand under control conditions (left) and during washout after prior superfusion with 20  $\mu\text{mol/l}$  PA (right). Thick arrows denote multiple notches in the upstroke of one of the signals. APA = AP amplitude. Reproduced from Rohr et al. [21] with permission.

with only minimally decreased  $dV/dt_{\text{max}}$ . The ultraslow conduction demonstrated with reduction in gap junctional coupling suggests the feasibility of microentry over very small distances.

While cellular uncoupling can impair AP propagation thereby leading to slow conduction, conduction block and reentry [23], structural discontinuities (whether due to discrete interconnections between muscle layers, to the Purkinje fiber ventricular junction or to surviving strands of viable myocardial cells in an infarct zone [24–26]) can also cause altered propagation. In these cases, a small current source connected to a large current load results in current-to-load mismatch that ultimately causes slow conduction and block.



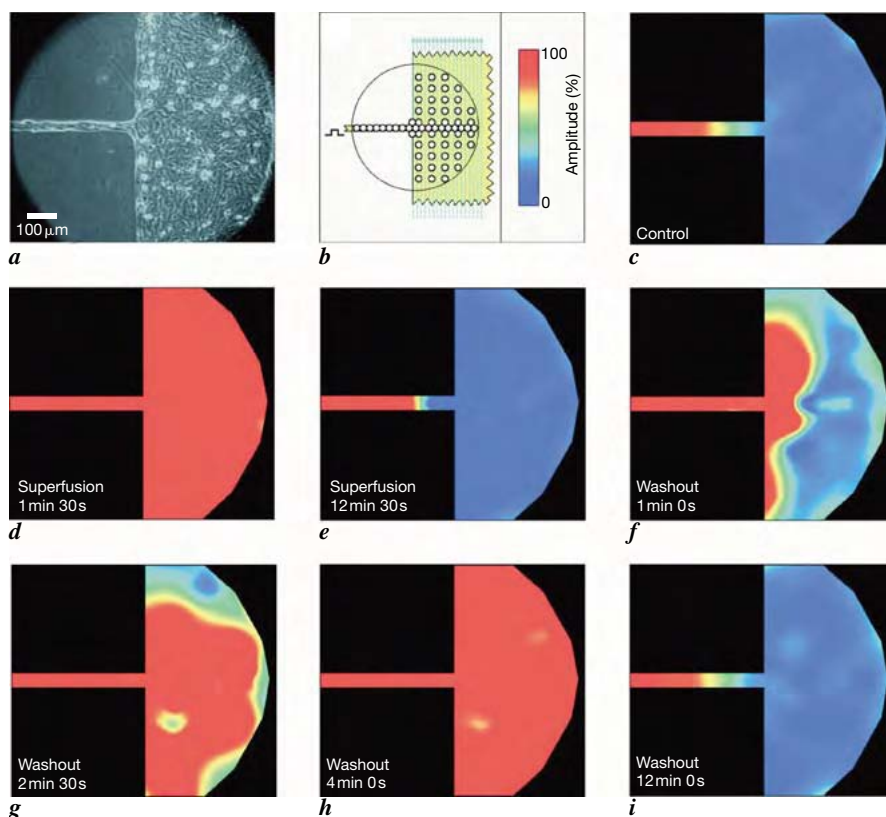


While both can contribute to arrhythmogenicity, it has been assumed that they act synergistically. However, intriguing work by Rohr et al. [27] demonstrated that uniform electrical uncoupling in the setting of structural discontinuities actually improved conduction. Here, these investigators expanded on their prior work with narrow cell strands connected to rectangular cell monolayers (expansions, fig. 6a, b). Under control conditions, antegrade block was evident by the attenuation of depolarizing current into the expansion (fig. 6c). However, microsuperfusion of palmitoleic acid (an uncoupler that does not alter AP [28]), confined to the area of expansion, gradually induced successful antegrade conduction (fig. 6d). With further superfusion, the expansion became uncoupled with unidirectional block at the border of the superfusion (fig. 6e). Moreover, with washout of palmitoleic acid, there was progressive recoupling where activation captured increasingly larger areas of the expansion (fig. 6f–h), after which unidirectional block was reestablished (fig. 6i). The concentric expansion of activation suggested a spatially uniform degree of uncoupling that could be explained by an increase in internal resistance ( $r_i$ ) by palmitoleic acid that resulted in a homogeneous decrease in load, thereby improving current-to-load mismatch. Thus the effects of uncoupling on load predominated over effects on the source. Computer simulations suggest that this may be due to curved activation wavefronts in the expansion, which may be more sensitive than the highly parallel current flow in the strand. These results imply that, in the setting of structural discontinuities (e.g. disease states), the nonsymmetric effects of uncoupling on current sources and loads could at times have antiarrhythmic effects by improving conduction.

### Studies in Mice with Targeted Deletions in Cx43

Cx43 is the most abundant connexin protein in ventricular myocytes [29]. Genetically engineered mice with targeted deletions of Cx43 have decreased

**Fig. 5.** Impulse propagation in normal and uncoupled cell strands. **a, b** Normal cellular coupling is evident in the phase contrast image of a cell strand (**a**) with white lines denoting evenly spaced isochrones with rapid activation. This is also evident by rapid successful optical AP upstrokes (**b**). The white circles in **a** denote the location of individual photodetectors. **c–e** Cellular uncoupling with palmitoleic acid in a cell strand. **c** Phase contrast image as in **a**. **d** With left to right propagation, optical AP upstrokes are clustered with large delays between clusters reflecting very discontinuous conduction. **e** Schematic diagram of the cell strand in **c** with activation pathways denoted by the arrows. The areas that have near simultaneous activation are numbered as in **c**. Hatched regions denote completely uncoupled cells. APA = AP amplitude. Reproduced with permission from Rohr [58].



**Fig. 6.** Optical mapping of abrupt tissue expansion (narrow strand connected to a large rectangular cell monolayer in patterned growth myocyte cultures (stained with di-8-ANEPPS). **a** Videomicrograph. **b** Schematic of a preparation (stimulated antegradely from left at 2 Hz) with circles denoting individual photosensors and the blue lines depicting regions undergoing local superfusion with palmitoleic acid. False-color coding reflects maximal signal amplitude with dark blue representing absence of depolarization and red representing maximal signal amplitude. **c** Under control conditions, there is antegrade conduction block. **d** With partial uncoupling (palmitoleic acid superfusion), there is successful antegrade conduction. **e** After prolonged palmitoleic acid superfusion, there is complete uncoupling of the expansion. **f–h** With progressive washout of palmitoleic acid, there is capture of larger regions of the expansion before full successful antegrade activation. **i** After prolonged palmitoleic acid washout, there is reestablishment of antegrade conduction block. Reproduced with permission from Rohr et al. [27].

expression of this protein in vivo, and have been extensively used to study the effects of decreased gap junctional coupling on arrhythmogenesis. Homozygous null mice (Cx43<sup>-/-</sup>) exhibit significant cardiac abnormalities including obstruction of the right-ventricular outflow tract, and die soon after birth [30, 31].

Heterozygote (Cx43 $+/ -$ ) mice, which have a 50% decrease in Cx43 protein expression (with no change in Cx40 or Cx45 expression), have normal cardiac morphology and are able to breed [32–35]. They do not die prematurely, but they exhibit an increased incidence and duration of ventricular arrhythmias during myocardial ischemia [36], suggesting that moderate alterations in cellular coupling may require additional insults (e.g. ischemia, structural remodeling or electrical remodeling) for arrhythmogenesis to develop. Assessment of conduction velocity in Cx43 $+/ -$  mice has yielded conflicting results, with several studies demonstrating significantly decreased conduction velocity (along with prolonged duration of the QRS complex) [32, 33, 35] while other studies failed to demonstrate differences in conduction velocity [34, 37].

Recently, Thomas et al. [38] studied electrical properties of synthetic cell strands of cultured neonatal myocytes from Cx43 $+/ -$  mice and found no difference in conduction velocity compared to control myocytes. However, they did find increased  $dV/dt_{\max}$  and reduced AP duration in Cx43 $+/ -$  cell strands. Computer modeling suggested that the unchanged conduction velocity was explained by both a dominant role of myoplasmic resistance (over gap junctional resistance) on axial resistance and a compensatory increase in  $dV/dt_{\max}$  due to upregulation of  $I_{Na}$  (although Johnson et al. [39] have reported no change in  $I_{Na}$  in myocytes from Cx43 $+/ -$  mice).

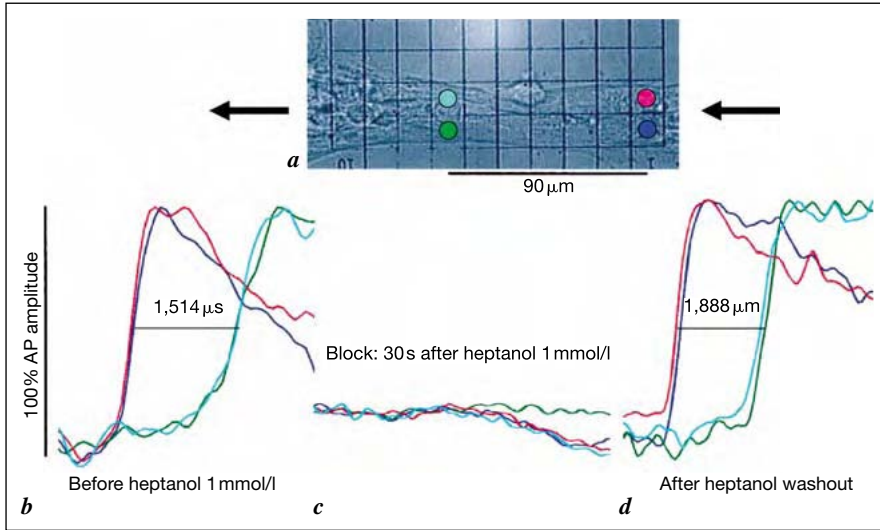
### **Cardiac-Specific Connexin43 Knockouts**

To get around the problem of perinatal mortality, Gutstein et al. [40] have developed a cardiac-restricted knockout of Cx43 (CKO mice, where cardiac Cx43 is deleted during development) that has normal heart structure and contractile function. These mice exhibit a 95% decrease in Cx43 expression compared to littermate controls and they uniformly develop sudden death from spontaneous ventricular arrhythmias by 2 months of age. High-resolution optical mapping of epicardial activation has demonstrated slowing of conduction both in the transverse (up to 55%) and longitudinal (up to 42%) directions, and an increased anisotropic ratio (2.1 vs. 1.7) compared to control littermates [40].

Yao et al. [41] evaluated cell coupling in ventricular myocyte pairs from CKO and control mice by measuring gap junctional conductance using a dual patch clamp technique. Immunofluorescence studies revealed that  $\sim 10\%$  of CKO myocytes expressed normal levels of Cx43. This raises an important question as to whether it is the heterogeneous Cx43 expression (rather than the mere decrease in abundance) that might play a key role in the development of arrhythmogenesis in this model. While the above optical mapping studies had

shown that conduction velocity in CKO hearts were decreased by  $\sim 50\%$ , patch clamp studies showed that the majority of myocyte pairs from CKO mice had markedly reduced gap junctional conductance (4 and 11 nS for side-to-side and end-to-end cell pairs, compared to 588 and 558 nS in controls, respectively). Yao et al. [41] proposed a number of potential mechanisms that might account for such a disparity between the moderate slowing of conduction velocity and the dramatic decrease in gap junctional conductance including: the contribution of connexins other than Cx43, effects with enzymatic digestion of cells, connexin internalization [42–44],  $I_{Na}$  upregulation and the influence of  $I_{Na}$  and cleft currents [45], and the small diameter of murine myocytes which makes axial resistance more dependent on myoplasmic resistance than on gap junctional resistance [38]. Another potential mechanism proposed was the electric field mechanism of AP propagation that could mediate transmission in the absence of intercellular coupling by gap junctions. Sperelakis [46] and Sperelakis and McConnel [47] have proposed that activation of  $Na^+$  channels in the activated cell shifts the cleft potential within the intercalated disks between two abutting cells in the negative direction, and this shift in potential depolarizes the  $Na^+$  channels within the intercalated disc of the neighboring resting cell. While based primarily on a mathematical model, the hypothesis may explain several experimental observations, including the above by Yao et al. [41]. In this regard, recent findings that the  $Na^+$  channels are located mainly within the intercalated disks, where they can create sufficient local circuit current to shift the ‘cleft potential’ sufficiently to activate the  $Na^+$  channels in the neighboring cells, are intriguing [48–50]. However, the hypothesis cannot accommodate the conduction through the SA or AV node (where sodium currents do not play a dominant role). Also difficult to reconcile with this hypothesis (which does not depend on low-resistance coupling between the cells) are numerous observations by multiple investigators on the cable-like properties of Purkinje fibers based on the ‘source-sink’ principles. A recent model proposed by Kucera et al. [45] may offer a combined view of interplay between clustering of the  $Na^+$  channel and gap junctions in the intercalated disks. Results in their model, primarily based on the ‘classical’ concept of low-resistance cellular coupling, suggest that clustering of  $Na^+$  channels ‘modulate’ the conduction and even support it when cellular coupling is significantly diminished.

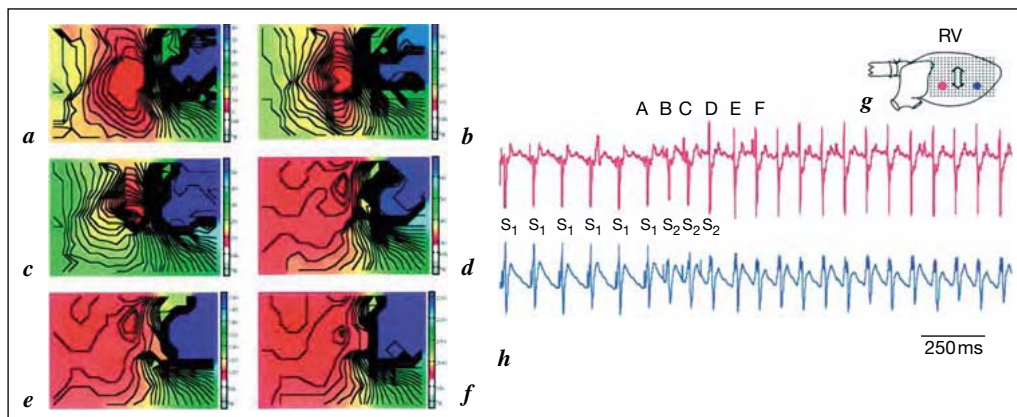
Relevant to this issue, Yao et al. [41] found that the conductance of CKO cell pairs shows increased voltage dependence suggesting the presence of other connexins at low levels. They also demonstrated that the small residual gap junctional conductance in CKO cell pairs was completely blocked by the gap junction inhibitor halothane, implying that the remaining conductance is dependent on communication via gap junction channels rather than alternative mechanisms such as electric fields in junctional clefts [46, 47].



**Fig. 7.** Propagation in cell strands from Cx43<sup>-/-</sup> mice. **a** Micrograph of a synthetic strand with circles depicting positions of selected measuring photosensors. The two circles at the right and left correspond to the early and late AP upstrokes, respectively, clustered below. **b–d** Discontinuous propagation evident by AP upstrokes with nearly simultaneous excitation within individual myocytes but marked conduction delay ( $>1,500 \mu\text{s}$ ) between cells (**b**). Superfusion of the uncoupler heptanol led to block of propagation (within 30 s, **c**), which recovered after washout of heptanol (**d**). Reproduced with permission from Beauchamp et al. [51].

Similar conclusions were drawn by Beauchamp et al. [51] in their studies of cultured ventricular myocyte strands and cell pairs from germline Cx43<sup>-/-</sup> mice. They found no immunoreactive signal for Cx43 but persistence of Cx45 protein levels by Western blotting. Gap junctional conductance in Cx43<sup>-/-</sup> cell pairs was reduced by 96% and propagation in cell strands was very slow and discontinuous (fig. 7b). However, this slow propagation was completely abolished by heptanol (fig. 7c), but returned during washout (fig. 7d), indicating residual gap junctional coupling and ruling out field effect transmission as a potential mechanism.

To further explore this issue, Danik et al. [52] performed studies in a genetically engineered line of mice with cardiac-specific knockout of Cx43 expressing gradually decreasing levels of Cx43 (O-CKO) without compensatory changes in the abundance of either Cx45 or Cx40. These mice demonstrate prolonged survival (vs. CKO mice) but still die prematurely compared to controls. By 25 days of age, O-CKO mice demonstrate a 59% decrease in Cx43



**Fig. 8.** Optical recording of induced sustained VT on the right ventricle of a conditional Cx43-deficient mouse. **g** Schematic of the heart with the grid denoting sites mapped. **h** ECG tracings of an induced monomorphic VT and A-F denote VT beats whose maps are shown in **a-f**. **a-f** Maps of VT beats A-F showing very slow conduction (increased density of isochrones) leading to reentrant excitation. Reproduced with permission from van Rijen et al. [56].

protein levels with no change in conduction velocity and no inducible ventricular arrhythmias. However, by 45 days of age, Cx43 protein expression has decreased by 82%, conduction velocity has slowed by about half that of controls and nearly 80% of mice are inducible into ventricular tachycardia (VT) [52]. These results suggest that progressive and heterogeneous loss of Cx43 can lead to slow conduction and increased susceptibility to ventricular arrhythmias.

Very recent optical mapping studies in these O-CKO mice by Morley et al. [53] demonstrated multiple sites of epicardial breakthrough during sinus rhythm compared to controls but comparable conduction velocities during ventricular pacing. They proposed that uncoupling of gap junctions enhances propagation across Purkinje-ventricular junctions (that may normally be quiescent) and result in wavefront collisions that could initiate reentry, as predicted by modeling studies of Joyner et al. [54].

### Inducible Model of Connexin43 Gene Deletion

In order to define the contribution of gap junction channels to conduction in the adult heart, Eckardt et al. [55] recently described a novel mouse model targeting the 4-hydroxytamoxifen (4-OHT)-inducible Cre recombinase into the endogenous Cx43 locus. 4-OHT ablation of Cx43 in adult mice led to a

65–95% decrease in Cx43 protein without significant upregulation of Cx45 or Cx40. There was significant prolongation of the QRS duration. Moreover, these mice died within 3 weeks after their last induction, and telemetric ECG monitoring in 3 mice demonstrated the occurrence of polymorphic VT in all. High-resolution electrode mapping (300- $\mu$ m resolution) revealed that these mice exhibited slow conduction in both the longitudinal and transverse directions, and an increased anisotropic ratio. Ectopic beats and runs of sustained VT were induced in 70% of these mice, and maps suggested VT was initiated by reentry, most commonly in the right ventricle (fig. 8) [56]. In this model, as well as the CKO model above, it is likely that the combination of decreased Cx43 expression combined with patchy residual expression ultimately created the substrate for arrhythmogenesis.

## Conclusions

Gap junctions play a significant role in AP propagation, with microscopic conduction in the ventricle involving longitudinal and transverse spread of the propagating wavefront via gap junctions.

However, conduction velocity is also affected by other features such as structural discontinuities and excitability (primarily involving Na and Ca currents). Studies of microscopic conduction in patterned cultures of neonatal rodent myocytes have provided novel insights into the role of gap junctions, the effects of uncoupling versus altered excitability, and the contribution of discontinuities and branching. Decreased gap junctional coupling can contribute to slowing of conduction and development of unidirectional block. However, in the setting of structural inhomogeneities and unbalanced current source and load, decreased coupling can, at times, improve conduction and be ‘anti-arrhythmic’, attesting to the complexity of intercellular coupling as a therapeutic target.

Genetically engineered mouse models of Cx43 depletion have also provided novel insights into the contribution of gap junctions to impulse propagation and arrhythmogenesis in the intact heart. Studies in models with markedly reduced Cx43 expression demonstrate slow conduction and arrhythmogenesis that appears to be reentrant in nature. The underlying role of diminished Cx43 expression versus the heterogeneity of Cx43 expression requires further study. While the persistence of very slow conduction in the setting of dramatic downregulation of Cx43 raises the possibility of the electric field theory, the experimental data to date support residual gap junctional conductance, ruling out the electric field theory as an alternative mechanism.

Overall, gap junction expression, distribution and heterogeneity are important contributors to microscopic conduction, and alterations in any of these (as well as in



the phosphorylation of connexin proteins [57]) can contribute to the development of an arrhythmogenic substrate in pathological states. Experimental studies of micro-conduction (combined with mathematical modeling) have increased our understanding of the complexity of AP propagation. They have also brought us closer to developing therapeutic approaches to modulate cellular coupling in order to prevent and treat malignant ventricular arrhythmias arising from altered conduction.

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## Pharmacology of Cardiovascular Gap Junctions

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### Abstract

Gap junction (GJ) channels play an important role in forming a functional network or syncytium of cells by allowing the transfer of small molecules or the conduction of electrical activation. These channels can be regulated at the level of acute opening or closure as well as at the level of expression including synthesis, protein trafficking and degradation. Many of the underlying mechanisms depend on phosphorylation or dephosphorylation of connexins. A number of drugs is available to study GJ function and connexin expression. Some of these drugs have shown therapeutic effects, e.g. the anti-arrhythmic peptides AAP10 and ZP123 in the prevention of certain types of arrhythmia. Moreover, mediators involved in cardiovascular pathophysiology, e.g. angiotensin, endothelin, tumor necrosis factor- $\alpha$ , fibroblast growth factor and others, affect connexin expression and can alter the Cx43/Cx40 ratio, which may contribute to the formation of an arrhythmogenic substrate. On the other hand, drugs affecting these mediators may influence GJ networking and may thus open new therapeutic horizons.

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Gap junctional (GJ) channels are dynamic plasma membrane structures with a high turnover rate. The half-life of Cx43, for example, was reported to range between 1 and 3 h, faster than most other integral membrane proteins, but of the same order as half-lives reported for channel-forming proteins of some other membrane channels (e.g. Kv1.4 K<sup>+</sup> or epithelial Na<sup>+</sup> channels [1]). Protein phosphorylation is regarded as the main mechanism of regulation of GJ communication, governing connexin trafficking from the Golgi complex to the plasma membrane, aggregation of channels into selected areas and prevention of their free diffusion throughout the lipid bilayer, removal from the plasma membrane, degradation, as well as the gating of GJ channels. A large range of signaling molecules might then, via a spectrum of protein kinases (PKs) and

phosphatases (PPs), be able to cause rapid and reversible changes capable of modulating the GJ intercellular communication (GJIC), leading to the hypothesis that junctional channels undergo conformational changes that gate the channels, either directly or via interactions involving discrete domains of Cx43. After modification of phosphorylation on a particular site, the C-terminal region of the connexin might for example interact either with the pore-forming region of the channel or with an intermediary molecule, to form a complex resulting in a channel pore closure. Since phosphorylation and dephosphorylation are often involved in the pharmacological effects on acute or chronic regulation of GJIC, we will first give an overview on this issue.

In mammals, cardiac cells predominantly express junctional channels built of Cx40, Cx43 and Cx45. The three connexins not only contain PK consensus phosphorylation sequences but also have been demonstrated to be phosphorylated by PKs *in vitro*, and in some cases in cultured cells or tissues, to exhibit different electrophoretic mobility and to be able to incorporate  $^{32}\text{P}$  [2]. Phosphorylation occurs on serine and, in some cases, on tyrosine residues.

### **Enzymes Catalyzing Protein Phosphorylation in Cardiac Tissues**

Among the investigated PKs, cAMP-activated PK (PKA), PKC, p34<sup>cdc2</sup>, casein kinase 1, mitogen-activated PK (MAPK) and a tyrosine PK encoded by the viral oncogene v-src (pp60<sup>v-Src</sup>) have been shown to target connexins [for reviews, see ref. 2, 3]. The main potential phosphorylation sites present in the sequence of the C-terminal tail of rat cardiomyocytes Cx43 are presented in table 1.

#### *Protein Tyrosine Kinases*

Exposure of ventricular cardiac myocytes to the tyrosine PK inhibitors genistein [4] or 4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo-[3,4-*d*]-pyrimidine [5] had no effect on the intercellular dye diffusion, whereas tyrosine PK activation usually results in decreases in GJIC [for review, see ref. 6]. Genistein prevented the GJIC disruption caused by fibroblast growth factor-2 (FGF-2) between cardiomyocytes [4].

#### *Protein Serine/Threonine Kinases*

Cardiac ventricular myocytes exposed to factors that promote cAMP-dependent protein phosphorylation (e.g. externally applied 8-bromo cAMP or internally applied cAMP) did not exhibit changes in the strength of acute GJ coupling [7, 8]. Such a result is consistent with the fact that ATP alone is

**Table 1.** Potential sites of phosphorylation in protein sequence of the C-terminal tail of rat cardiomyocytes Cx43

Position	Enzymes	Cell types	References
S373	unidentified	rat primary granulosa cells	♠ 2
S372	PKC	newborn rat cardiomyocytes	101
S369	unidentified	rat primary granulosa cells	♠ 2
S368	PKC	newborn rat cardiomyocytes	101, 102
	PKC	mouse fibroblasts	103
	unidentified	rat primary granulosa cells	♠ 2
S365	unidentified	rat primary granulosa cells	♠ 2
S364	PKA dependent	murine cell lines	♦ 104
	PKA	murine fibroblasts L929	105
S330	CK1	normal rat kidney cells	20
S328	CK1	normal rat kidney cells	20
S325	CK1	normal rat kidney cells	20
S282	MAPK	T51B rat liver epithelial cells	106
		Cx43-HeLa cells	107
S279	MAPK	T51B rat liver epithelial cells	108
		T51B rat liver epithelial cells	106
		Cx43-HeLa cells	107
Y265	pp60 <sup>v-Src</sup>	Xenopus oocytes	109
		murine cell lines	110
S262	p34 <sup>cdc2</sup>	Xenopus oocytes	111
S257	PKG	human hepatoma SKHep1 cells	16
S255	p34 <sup>cdc2</sup>	Xenopus oocytes	111
		Rat1 fibroblasts, T51B rat liver	♣ 112
	MAPK	epithelial cells	108
		T51B rat liver epithelial cells	106
		T51B rat liver epithelial cells	107
	MAPK (MEK5-BMK1)	Cx43-HeLa cells	♥ 113
		Cx43 human embryonic kidney (HEK) 293 cells	
Y247	pp60 <sup>v-Src</sup>	murine cell lines	1

♠ = Induced by FSH, known to increase cAMP concentration, the effect might result from PKA activation or be due to a PKA-independent mechanism; ♣ = possibly via a downstream unidentified PK activated by pp60<sup>v-Src</sup>/cyclin B PK; ♦ = possibly by mediating an interaction with intracellular trafficking protein; ♥ = S255 appears, in these conditions, to be the only BMK1 phosphorylation site on Cx43 in vivo.

sufficient to prevent the loss of activity of junctional channels (channel ‘run-down’) in whole cell patch clamp configuration. In contrast, Mg-ATP fails to prevent the loss of activity of several other channels (both voltage or ligand activated) except when the purified catalytic subunit of PKA is co-added. In these cases, a membrane-bound PK involved in the phosphorylation of the channel, or at least its catalytic subunit, might have been lost after patch excision. In excised cardiac myocytes, the addition of the catalytic subunit of PKA had no effect on GJ conductance [7]. Cx43 protein was furthermore shown to be a poor substrate of PKA and, on the other hand, evidence has been accumulating that cAMP may act via a PKA-independent mechanism in addition to PKA-dependent pathways [9].

Several inhibitors of PKC activity, such as staurosporine or calphostin C [10] or chelerythrine [11], had no effect on the degree of cell-to-cell dye coupling between neonatal rat cardiomyocytes. However, PKC activation might enhance the junctional coupling: a 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA)-induced increase in macroscopic GJ between neonatal rat cardiomyocytes was ascribed to PKC activation [1, 12] and was indeed prevented when calphostin C was present [Lallouche and Hervé, unpubl. obs.]. In the same cell type, Cx43 rephosphorylation after ischemia was impeded by broad-spectrum PKC inhibitors, whereas in normoxic cells prolonged treatment (1 h) with a PKC inhibitor had no effect on the Cx43 phosphorylation status [13]. In this preparation, PKC might be involved in the upregulation of the channel rather than in determining the basal degree of protein phosphorylation, which governs channel activity. In cardiomyocytes, the enhancement in GJ conductance caused by the antiarrhythmic peptide AAP10 was shown to result from PKC $\alpha$  activation [14]. Chelerythrine prevented both the reduction in dye coupling and the increased Cx43 phosphorylation caused by FGF-2 in cardiomyocytes. It was also observed that FGF-2 increased the co-localization of Cx43 with a PKC isoform (PKC $\epsilon$  [11]) resulting in decreased coupling. Activation of PKC by phorbol esters, e.g. TPA, was described in some studies to enhance macroscopic GJ conductance [15, 16], while there was no effect in other studies [17]. On the other hand, uncoupling effects of PKC activators have also been described [18]. In cardiac tissue, several isoforms of PKC are expressed, including PKC $\alpha$ , PKC $\beta$ , PKC $\epsilon$ , PKC $\xi$  and PKC $\gamma$  (rabbit heart [19]). However, only PKC $\gamma$  was found to be located close to the intercalated disks in this study. TPA treatment is assumed to result in a rapid translocation of PKC $\alpha$  and PKC $\epsilon$  in cultured neonatal rat cardiac myocytes [8]. Thus, one may argue that not all isoforms contribute to the GJ regulation and that differences between various preparations or tissues may depend on the subtypes of PKC involved. Since TPA activates both isoforms (which seem to exert opposite effects on GJ conductance) the diverging results obtained with TPA reported in the literature might be

explained by a different PKA $\alpha$ /PKC $\epsilon$  activation ratio. The conflicting results regarding PKC influence, thus, might be due to differences in PKC isoforms, in Ca<sup>2+</sup> concentration in the pipette solution or in the connexin phosphorylation status prior to treatment; it was also suggested that PKC does not directly phosphorylate connexins but may activate other regulatory proteins [12].

Treatments (up to 30 min) with inhibitors of protein kinases CK1 and 2 (5,6-dichlorobenzimidazole 1- $\beta$ -D-ribofuranoside), glycogen synthase kinase 3 (LiCl), Ca<sup>2+</sup>/CaM type-2 PK (KN93) failed to alter the degree of cell-to-cell communication between rat ventricular myocytes significantly [Plaisance et al., unpubl. obs.]. Longer exposures (2–4 h) to a CK1-specific inhibitor (CKI-7) reduced the phosphorylation level of phosphorylation Cx43, altered its localization and decreased the cell-to-cell dye transfer [20]. Some decreases in the strength of intercellular coupling between cardiac cells were also ascribed to the activation of different PKs. Two examples are given in the following: (i) cGMP-activated PK (PKG), whose activation led to a GJ decrease (–26%) in newborn rat cardiomyocytes [8], and (ii) under pathological stress (such as hypertension, ischemia/reperfusion injury or myocardial infarction), a number of intracellular signaling pathways are activated in ventricular myocytes, including c-Jun N-terminal kinase (JNK), a stress-activated PK which, along with p38 and extracellular signal-regulated kinases, constitutes the highly conserved MAPK family. JNK signaling appears as a key mediator of pathological cardiac remodeling and heart failure. JNK activation led to specific loss of Cx43 and GJs and impaired GJIC without affecting the expression or localization of other key intercalated-disk proteins [21].

### **Enzymes Catalyzing Dephosphorylation in Cardiac Tissues**

In mammalian hearts, PP1 and PP2A were found to account for the main part of the PP activities; in the human heart for example, all PP1 and PP2A mRNA transcripts were expressed in both right and left ventricles (PP1 $\alpha$  > PP1 $\gamma$  > PP1 $\delta$  and PP2A $\alpha$  > PP2A $\beta$ ), but PP expression at the protein level remains to be investigated. PP2B has been implicated in transduction signals responsible for inducing cardiac hypertrophy whereas PP2C isoenzymes seem to be confined to human, rat and mouse heart tissues, where they might subserve special functions. Among the new mammalian phosphoserine/phosphotyrosine (PT) PPs, PP4, PP5 and PP6 have been observed in heart tissues and PTPP activities have been identified in bovine, rat and murine heart tissues [1]. In cell-free assays, P1 and PP2A activities, both sensitive to inhibitors such as okadaic acid and calyculin A, can be distinguished using appropriate concentrations of these inhibitors, but the situation is different in intact cells, where PP1 and PP2A



are present at concentrations approaching the micromolar range; 1  $\mu$ M okadaic acid is thereby required to inhibit these PPs totally, and selective inhibition of PP2A versus PP1 is no longer possible [1].

In normal rabbit cardiomyocytes, low okadaic acid (10 nM) had no effect on the cell-to-cell dye transfer whereas it substantially increased this diffusion between myocytes from an animal model of non-ischemic heart failure [22]. At this okadaic acid concentration, a part of PP2A activity is expected to be inhibited. In rabbits, non-ischemic heart failure myocytes exhibited reduced intercellular coupling together with decreased Cx43 expression and enhanced (over 2.5-fold) Cx43-PP2A co-localization whereas co-localized Cx43-PP1 remained unchanged. It was suggested that decreased Cx43 expression and phosphorylation might contribute to the altered conduction in cardiopathic rabbits and humans [22]. In rat ventricular myocytes, all treatments (such as ATP removal, exposure to a respiratory inhibitor or to a broad-spectrum inhibitor of endogenous serine/threonine PKs, introduction of an exogenous PP into the cells or activation of endogenous PP1) able to shift the protein phosphorylation/dephosphorylation balance towards dephosphorylation led to disruption of the junctional communication, an effect mainly ascribed to PP1 activities [23], but this junctional uncoupling frequently occurs without modification of the Cx43 phosphorylation profile [24]. In the same preparation, the reduction in the intracellular level of ATP by hypoxia, metabolic inhibition or simulated ischemia induced a progressive Cx43 dephosphorylation, which the presence of inhibitors of PP1, PP2A or PP2B failed to prevent [13].

In both isolated adult cardiomyocytes and isolated perfused hearts of the rat, simulated ischemia caused Cx43 dephosphorylation, being less important when okadaic acid or calyculin A was present. As fostriecin, a selective PP2A inhibitor, failed to prevent Cx43 dephosphorylation, the latter was ascribed to PP1 activity [25].

### **Influence of Protein Phosphorylation on Hemichannel Permeability**

Hemichannels present in the non-junctional regions of the plasma membrane, viewed for a long time as plasma membrane precursors of intercellular channels, are normally kept closed in the presence of normal extracellular  $\text{Ca}^{2+}$ , but it was recently reported that different cells might tolerate some hemichannel openings, which might exert physiological or deleterious effects, depending on the situation.

In rabbit ventricular myocytes, metabolic inhibition allowed loading of cells with a fluorescent dye (calcein) through open Cx43 unapposed hemichannels in

the presence of normal extracellular  $\text{Ca}^{2+}$  concentration [26]. A similar observation was made in other cell types (e.g. rat or mouse cortical astrocytes), with simultaneous reduction in intracellular ATP level and Cx43 dephosphorylation ascribed to PP2B (calcineurin) activity [1]. In cardiac tissues, if activated during ischemia, such hemichannel openings could significantly contribute to altered ionic fluxes promoting arrhythmias and myocardial injury [26].

### **Protein Substrate of Phosphorylation/Dephosphorylation**

The fact that some dephosphorylating treatments are able to impair cell-to-cell coupling without noticeable change in the Cx43 band pattern and, conversely, that changes in the connexin phosphorylation level caused by exposure to different compounds occurred without alteration in the GJIC degree, however, show that the mechanisms controlling the functional state of junctional channels are probably more complex than a direct connexin phosphorylation/dephosphorylation and plausibly involve regulatory proteins important for the modulation of the channel activity.

An illustration of the signal transduction cascade was given by the effects of FGF-2 (an endogenous growth-promoting protein believed to be involved in the regulation of hyperplastic and hypertrophic growth, cardiogenesis and angiogenesis [4]), known to activate receptors linked to PKC and to MAPK. FGF-2 reduced the cell-to-cell dye coupling between neonatal rat cardiomyocytes and stimulated Cx43 phosphorylation [4, 11]. These effects were observed in the presence of an MAPK inhibitor (PD98059) whereas PKC inhibition by chelerythrine blocked them [11]. However, they were also prevented by genistein, a tyrosine phosphorylation inhibitor [4]. In the latter study, phosphoamino acid analysis did not detect tyrosine phosphorylation on Cx43, but since a fraction of Cx43 was immunoprecipitated with anti-phosphotyrosine-specific antibodies after FGF-2 treatment, an interaction of Cx43 with a phosphotyrosine protein was suggested. The dye diffusion between Cx43-HeLa cells was severely reduced when S365, S368, S369 and S373 (four FSH-inducible phosphorylation sites) were substituted together with alanine, whereas it was retained or only slightly reduced after a single mutation at each of these sites, suggesting that the phosphorylation of any single serine residue was not crucial for Cx43-mediated dye coupling [9].

In different cell types (e.g. astrocytes, osteoblasts and fibroblasts), a similar lack of correlation between the modification in the Cx43 band pattern and changes in GJIC was observed. The junctional uncoupling effects of the tumor promoter TPA are commonly ascribed to PK (particularly PKC) activation;

although Cx26 cannot be phosphorylated, TPA influences cell-to-cell dye transfer and junctional conductance in Cx26-expressing cells [24].

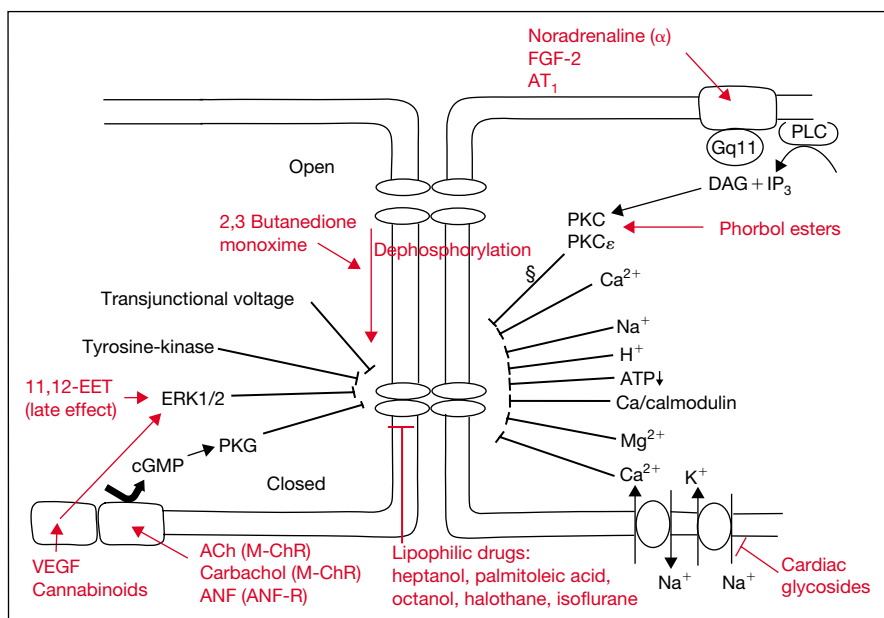
The degree of cell-to-cell communication, quantified for example by macroscopic GJ conductance, is determined by the number of channels, the conductance of a single channel and the open probability. Several channel blockers, such as heptanol, are considered to decrease the open probability without modification of the single channel conductance. Single channel conductance and open probability are very likely independently modulated by different mechanisms. In cells interconnected by Cx43-made channels, activation of protein phosphorylation frequently shifted the single channel conductances towards lower values whereas dephosphorylating treatments (e.g. exposure to PK inhibitors or to exogenous PPs [8, 16]) shifted them towards largest unitary conductances. In contrast, the latter treatments considerably lowered the macroscopic GJ. The increase in the elementary conductance might be due to a channel conformational change caused by connexin dephosphorylation whereas the open probability of the channels might be independently drastically reduced by such treatments. As recently discussed [1, 12], these observations led to the conclusion that, in addition to the Cx43 phosphorylation step itself, the GJIC modulation by protein phosphorylation/dephosphorylation events is probably more complex than direct connexin phosphorylation and may result from interactions with regulatory components. Direct association of several proteins with Cx43 and probable involvement of protein-to-protein interactions in the regulation of junctional channel activity are in accord with this hypothesis.

### **Agents for Acute Closure of Gap Junctions**

Several agents are reported that lead to acute closure of GJ channels within 5–10 min, which will be discussed in the following section. Figure 1 gives an overview of the acutely uncoupling agents.

#### *Ions and Drugs Affecting Ion Concentrations*

Some ions which can pass the channel are involved in the regulation of GJ conductance.  $\text{Ca}^{2+}$ , for example, reduces junctional permeability in cardiomyocytes [27, 28]. However, according to Noma and Tsuboi [28] considerably increased intracellular calcium concentrations ( $>1 \mu\text{mol/l}$ ) are needed to affect coupling in guinea pig hearts, while low changes in calcium do not affect GJ conductance in adult heart cells [29]. It was concluded [30] that a reduction in GJ conductance occurs if the intracellular calcium concentration exceeds the range of 320–560 nM. It has been suggested that the binding site for  $\text{Ca}^{2+}$  and also for  $\text{H}^{+}$  is located on the cytoplasmic loop of Cx43 [15]. Intracellular



**Fig. 1.** Closing GJ: scheme of agents and pathways that acutely (within minutes) disrupt GJIC. PKC $\epsilon$ : possible dependence on the isoform of the enzyme; transjunctional voltage/tyrosine kinase: divergent effects depending on species, cell type, or connexin isoform. ACh = Acetylcholine; ANF-R = atrial natriuretic factor receptor; DAG = diacylglycerol; IP<sub>3</sub> = inositol-1,4,5-triphosphate; M-ChR = M-cholinergic receptor; PLC = phospholipase C; rER = rough endoplasmic reticulum.

acidification is known to decrease junctional electrical coupling in cardiomyocytes and in Purkinje fibers [28, 31, 32]. GJ conductance was nearly constant in a pH range from 7.4 to 6.5 and decreased sharply when pH was reduced to 5.4 [28]. In neonatal rat heart cells, Firek and Weingart [33] found a pK<sub>H</sub> of 5.85. Cx45 channels seem to be more sensitive to pH than Cx43 channels [34]. Regarding the pH sensor, the carboxy tail length has been demonstrated as a determinant of pH sensitivity [35]. Further investigations [36] revealed that the carboxyterminus serves as an independent domain which can bind to another separate domain of the connexin protein closing the channel, comparable to the ball-and-chain model for potassium channels. In addition, increases in [Mg<sup>2+</sup>]<sub>i</sub> to 110 mM at pH 7.4 in the absence of calcium also reduce GJ conductance in pairs of adult guinea pig cardiomyocytes [28]. Na<sup>+</sup> also is involved in the regulation of GJ conductance: Na<sup>+</sup> withdrawal in adult rat cardiomyocytes induced

electrical uncoupling within 3 min [30], which might be a consequence of an impairment in the  $\text{Na}^+/\text{Ca}^{2+}$  exchange mechanism [30]. In addition, De Mello [37] described that an increase in  $[\text{Na}^+]_i$  caused uncoupling within 500 ms in Purkinje fibers which, on the other hand, might be secondary to a rise in intracellular calcium via the  $\text{Na}^+/\text{Ca}^{2+}$  exchange mechanism.

Pharmacologically, the intracellular concentrations of  $\text{Na}^+$  and  $\text{Ca}^{2+}$  can be affected by cardiac glycosides such as strophanthidin, ouabain, digitoxin or digoxin acting via inhibition of  $\text{Na}^+/\text{K}^+$ -ATPase leading to enhanced  $\text{Na}^+/\text{Ca}^{2+}$  exchange resulting in elevated intracellular  $\text{Ca}^{2+}$  [38]. This uncoupling occurs at concentrations of  $0.68 \mu\text{M}$  of ouabain, for example, which is near the therapeutic range [37] and thus may contribute to the well-known arrhythmogenicity of digitalis.

### *Lipophilic Agents*

Cardiac GJ channels can be uncoupled using micromolar concentrations of heptanol, octanol, myristoleic acid, decanoic acid or palmitoleic acid [39–41]. Most commonly, this is explained by an incorporation of these drugs into the lipid bilayer leading to impairment in the transcellular GJ channels. Heptanol reduces coupling by reducing open probability of the channels by a conformational change at the connexin-membrane lipid interface [42]. Heptanol has been shown to inhibit reversibly (within 2 min) GJ conductance with a  $K_D$  of  $0.16 \text{ mM}$ . Other agents are oleic acid, palmitoleic acid and myristoleic acid, which close GJ in neonatal rat cardiomyocytes with  $\text{EC}_{50}$  of about  $2\text{--}5 \mu\text{M}$  [39]. In whole heart Langendorff preparations of rabbit hearts, palmitoleic acid led to a more distinct impairment in transverse conduction by the fatty acid and concomitant increase in dispersion with an  $\text{EC}_{50}$  of  $3.3 \mu\text{M}$ , while there were no effects on the action potential at that concentration [41, 43]. Arachidonic acid also uncouples cells with a  $K_D$  of  $4 \mu\text{M}$  [44].

Inhalation anesthetics such as halothane or isoflurane have also been shown to interfere with intercellular coupling. Incubation of neonatal rat cardiomyocytes with  $2 \text{ mM}$  halothane resulted in a 90% reduction in initial GJ conductance within 15 s [45]. In a subsequent study, it was found that halothane reduced the mean open time while the mean closed time was increased, and single channel conductance remained unaffected [46].

Derivatives of glycyrrhizic acid such as  $18\text{-}\alpha\text{-glycyrrhetinic acid}$ ,  $18\text{-}\beta\text{-glycyrrhetinic acid}$  and carbenoxolone also uncouple GJ channels. Several authors have used  $18\alpha\text{-glycyrrhetinic acid}$  as a GJ inhibitor at concentrations of about  $50 \mu\text{M}$  [47] or  $18\beta\text{-glycyrrhetinic acid}$  at a concentration of  $5 \mu\text{M}$  [48]. It should be mentioned that these drugs (including carbenoxolone) are not specific for GJs. The effect of these compounds requires a longer exposure time than the above-mentioned drugs. The molecular mechanism of the glycyrrhizic

acid metabolites is still unknown and has been suggested to involve phosphorylation or changes in the aggregation of connexin subunits.

### *Receptor-Mediated Uncoupling*

Regulation of the cardiovascular system is maintained in the organism by mediators such as acetylcholine, noradrenaline and angiotensin (AT), besides many others which can also influence GJ conductance. Application of the acetylcholine analogue carbachol ( $100\ \mu\text{M}$ ) in neonatal heart resulted in a reduction in electrical coupling, which could be mimicked by 8-Br-cGMP [49] suggesting an action via PKG. The effect only occurred in whole cell patch configuration but not in perforated patch configuration, indicating that a cytosolic enzyme is necessary for the effect which is washed out by the pipette in the whole cell patch. The carbachol effect could be antagonized by alkaline phosphatase. However, from a pharmacological point of view, the physiological impact remains unclear since  $100\ \mu\text{M}$  is a considerably high concentration. The situation becomes even more obscure in the vasculature, where the responses to acetylcholine can be inhibited by GJ blockers [50]. Also, probably acting via cGMP, atrial natriuretic factor ( $10\ \text{nM}$ ) led to uncoupling in cell pairs isolated from cardiomyopathic hamsters [51].

Regarding the effects of adrenaline and noradrenaline, there are mainly investigations on the coupling-increasing effects of cAMP pathway stimulation via  $\beta$ -adrenoceptors. In adult rat ventricular cardiomyocytes,  $\alpha$ -adrenergic stimulation with phenylephrine decreases GJ coupling in a PKC-dependent manner [52]. Since  $\alpha$ -adrenoceptors couple via  $G_{q/11}$  proteins to the phospholipase C/inositol triphosphate/diacylglycerol/PKC pathway, it might be interesting to consider the effects of PKC activation on GJ communication.

AT has also been shown to influence GJ coupling in cardiac cells. The acute effects seem to be mediated via  $AT_1$  receptors coupled to  $G_{q/11}$  proteins and PKC. In adult ventricular cell pairs,  $1\ \mu\text{g/ml}$  AT-II rapidly decreased GJ conductance. In subsequent experiments, intracellular dialysis of  $10\ \text{nM}$  AT-I resulted in a decrease in GJ conductance which was completely inhibited by intracellular dialysis of  $1\ \text{nM}$  enalaprilat, demonstrating the possible existence of an intracellular AT-converting enzyme [53], which is supported by other findings [54].

The angiogenic vascular endothelial growth factor (VEGF) acting on tyrosine-kinase-coupled VEGF receptors (type 2) reversibly inhibits GJ dye transfer in endothelial cells within 15–30 min ( $50\ \text{ng/ml}$  VEGF) with a concomitant change in the phosphorylation of Cx43 [55].

Recently, it has been shown that 11,12-epoxyeicosatrienoic acid elicits an uncoupling effect in endothelial cells. This effect was biphasic: an initial improvement in interendothelial coupling was followed by a sustained uncoupling effect

which seemed to depend on activation of the extracellular-regulated kinases (ERK) 1/2 [56] suggesting the existence of an endogenous intracellular regulation of intercellular communication.

Among the eicosanoids, a thromboxane A<sub>2</sub> mimetic reduced dye transfer between human endothelial cells and led to internalization of Cx43 [57] associated with capillary formation.

Vascular GJIC can also be blocked using the cannabinoid receptor agonists  $\Delta^9$ -tetrahydrocannabinol (10–30  $\mu$ M) or the synthetic HU210 (10  $\mu$ M) which both led to Cx43 phosphorylation in an ERK1/2-dependent manner associated with a reduction in electrical coupling and dye transfer within 15 min in cultured endothelial cells [58].

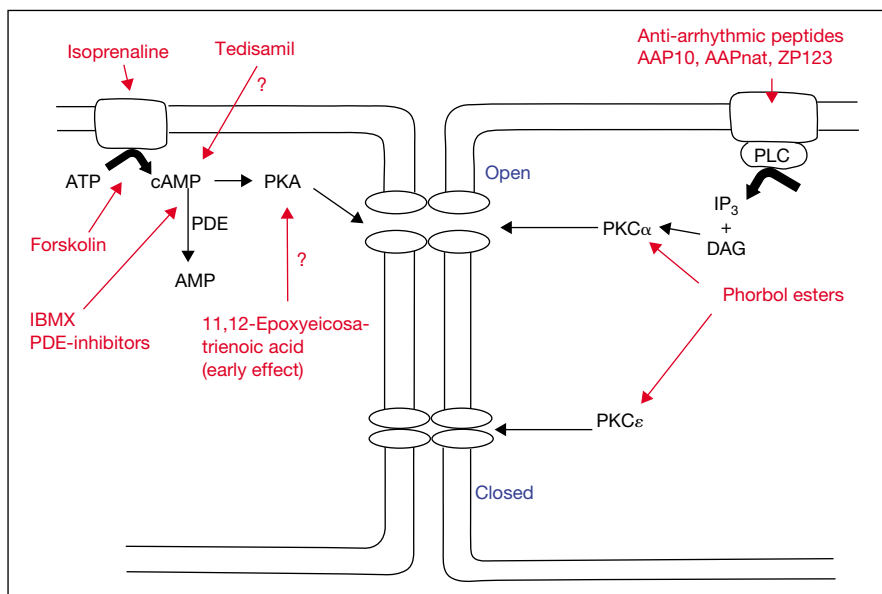
Recently, it was reported that fenamates reduce GJIC with an order of potency meclofenamic acid > niflumic acid > flufenamic acid and with IC<sub>50</sub> values of 25–40  $\mu$ M [59]. The exact molecular mechanism of action and a possible pharmacological meaning of this finding, however, is unclear at present.

### **Agents for Acute Opening of Gap Junctions**

Agents enhancing GJIC may be of particular interest since it has been shown that enhancing intercellular coupling can act anti-arrhythmically [60] especially in situations of reduced coupling such as ischemia-reperfusion. Figure 2 gives an overview on the agents available.

Among the mechanisms involved in enhancement of GJ coupling, PKA is discussed in a number of studies, but may be confined to Purkinje cells or cells coupled by Cx40 and Cx45 but not Cx43 (see above and the chapter by van Veen). It has been suggested that the effect of stimulation of the  $\beta$ -adrenoceptor/adenylylcyclase/PKA pathway may differ between normal heart and cardiomyopathic hearts due to alterations in this pathway. De Mello [61] showed in cardiomyocytes isolated from cardiomyopathic hamsters that isoprenaline, forskolin (a direct activator of adenylylcyclase) and isobutyrylmethylxanthine (an inhibitor of phosphodiesterases) failed to influence GJ conductance while in cells from normal hearts they increased GJIC. However, in both groups of cardiomyocytes dibutyryl-cAMP led to an increase in GJ coupling, showing that the  $\beta$ -adrenoceptor/adenylylcyclase system seemed to be downregulated or uncoupled while the cAMP/PKA system was still effective in controlling GJ conductance.

A new group of drugs, the anti-arrhythmic peptides (AAP), were first discovered in 1980 by Aonuma et al. [62], who identified a hexapeptide in bovine atria, designated AAP (AAPnat; H<sub>2</sub>N-Gly-Pro-4Hyp-Gly-Ala-Gly-COOH [63]), which improved synchronization of cultured myocardial cell clusters and



**Fig. 2.** Opening GJ: drugs and pathways improving GJIC acutely. Species differences exist for PKA. PKC $\alpha$  seems to be isoform dependent. Note the dual-faced effect of phorbol esters. DAG = diacylglycerol; IBMX = 3-isobutyl-1-methylxanthine; IP<sub>3</sub> = inositol-1,4,5-triphosphate; PDE = phosphodiesterase.

was shown in subsequent studies to possess anti-arrhythmic activity in various models [for a detailed overview, see ref. 60, 64]. While this peptide did not affect the transmembrane action potential, we could show that AAPnat and related synthetic derivatives (e.g. AAP10) increase GJIC [14, 65, 66]. Moreover, we found that the synthetic derivative AAP10 (H<sub>2</sub>N-Gly-Ala-Gly-Hyp-Pro-Tyr-CONH<sub>2</sub>) possesses a semicyclic structure and binds to a membrane protein which may serve as a membrane receptor [14, 67–69]. Since the AAP10 effect was sensitive to guanosine diphosphate- $\beta$ S and to PKC inhibitors as well as to a PKC $\alpha$ -specific inhibitor (CGP54345), it was concluded that AAP10 acts via a G-protein which downstream activates PKC $\alpha$  leading (directly or indirectly) to a phosphorylation of Cx43 resulting in an improvement in GJ conductance [14, 67]. It has been suggested that the AAP10 effect is more pronounced in cells which are partially uncoupled, e.g. by ischemia. It has been shown that AAP10 and AAPnat did not exert other effects on cardiac tissue and did not influence the cardiac action potential [65]. Thus, these peptides seem to be specific for GJs as far as we currently know. A more stable



derivative is the cyclopeptide cAAP10RG, i.e. c[CF<sub>3</sub>(OH)C-GAGHypPY] [68, 69] or the *D*-amino acid AAP10 analogue ZP123 (H<sub>2</sub>N-Gly-*D*-Ala-Gly-*D*-4Hyp-*D*-Pro-*D*-Tyr-Ac) [70]. Both drugs have been shown to reduce dispersion of action potential duration in a 256-electrode mapping in isolated rabbit hearts [71], which was not seen in the study by Kjolbye et al. [70], probably due to the low number of electrodes (8 electrodes) employed. All AAP10, AAPnat and ZP123 activate PKC [71]. ZP123 slowly increased GJ conductance in pairs of adult guinea pig cardiomyocytes [72], as was described for AAP10 [67]. Both AAP10 [69] and ZP123 [72] prevented from reentrant ventricular tachycardia.

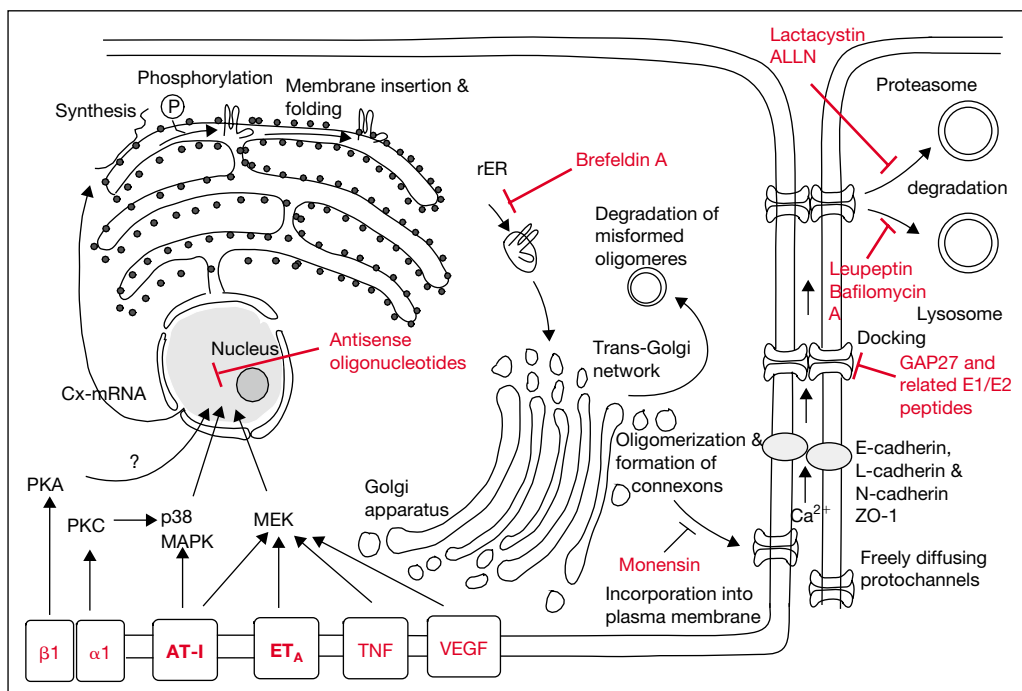
The antiarrhythmic agent tedisamil, a bradycardic drug, has been shown to increase GJ conductance by 58% (0.1  $\mu$ M) in cell pairs of cardiomyopathic hamsters [73]. Because of the sensitivity of the effect to inhibition of PKA, this was interpreted as a PKA-mediated action. However, it should be noted that tedisamil has also been reported to act on a number of other transmembrane ionic channels, such as sodium and potassium channels. Thus, tedisamil does not seem to be specific for GJs.

In the vasculature, hyperpolarization and vasorelaxation can be transmitted along the vessel via interendothelial GJs [74]. This may be related to the NO/PGI<sub>2</sub>-independent pathway coupled to the cytochrome P<sub>450</sub> isoform CYP2C and the generation of epoxyeicosatrienoic acids (EET) such as 11,12-EET [56]. Thus, as mentioned above, 3  $\mu$ M 11,12-EET had a biphasic effect on GJIC in human umbilical vein endothelial cells: 11,12-EET transiently enhanced GJIC within 1 min, followed by a prolonged uncoupling effect. Since the coupling action of 11,12-EET could be inhibited by a PKA inhibitor (KT5720) this increase in coupling may be PKA-mediated [56]. EETs serve as potent intracellular mediators and are involved in several signal transduction cascades, so that these observations may be of general interest.

Finally, the polyunsaturated  $\omega$ -3 fatty acid eicosapentaenoic acid has been assumed to enhance or preserve GJIC in endothelial cells: hypoxia/reoxygenation reduced GJIC in human umbilical vein endothelial cells after 2 h of reoxygenation which was inhibited by a 2-day pretreatment with 3  $\mu$ M eicosapentaenoic acid [75]. Eicosapentaenoic acid inhibited tyrosine phosphorylation of Cx43 induced by hypoxia/reoxygenation. Interestingly, under normoxia eicosapentaenoic acid had no effect on GJIC.

### **Agents Affecting Expression, Synthesis, Assembly, Docking and Degradation of Gap Junctions**

As already stated, connexins are subject to a considerably high turnover with half-lives of 1–5 h or in the case of Cx43 around 1.6 h, which allows the



**Fig. 3.** Regulation of connexin expression, synthesis, assembly, docking and degradation. Drugs interfering with these processes and thus affecting GJIC at a more chronic level are shown.

cells to adapt their communication to the actual situation. A number of substances and mediators can interfere with either connexin synthesis, formation, docking or degradation. Figure 3 gives a survey of the agents.

To suppress the expression of a specific connexin, it is possible to use oligonucleotides, as shown by Moore and Burt [76] who used 5'-GTCACC-CATGTCTGGGCA-3' as Cx43 antisense and 5'-GTCACCCATCTTGC-CAAG-3' as Cx40 antisense (24-hour treatment).

On the other hand, connexin expression can be induced, e.g. by the second messenger cAMP, as was shown for Cx45 and Cx43 after 24-hour treatment of cardiomyocytes with dibutyryl cAMP [77]. Accordingly, Salameh et al. [78] showed an upregulation of Cx43 in neonatal cardiomyocytes in response to forskolin, a direct activator of adenylylcyclase, indicating that activation of the adenylylcyclase/cAMP/PKA pathway can enhance Cx43 expression. Because in the cardiovascular system this pathway is typically activated by  $\beta$ -adrenergic

stimulation, this mechanism might play an important role in the process of adaptation of the heart to increased stress and higher heart rates.

Other physiologically relevant mediators [endothelin (ET), AT, tumor necrosis factor (TNF)  $\alpha$ , basic FGF (bFGF) and VEGF] can also affect connexin expression. ET-1 and AT-II increased Cx43 expression (ET-1:EC<sub>50</sub>: 158  $\pm$  41 nM, and AT-II:EC<sub>50</sub>:57  $\pm$  10 nM) and phosphorylation (ET-1:EC<sub>50</sub>: 13  $\pm$  3 nM, and AT-II:EC<sub>50</sub>:93  $\pm$  8 nM) in neonatal rat cardiomyocytes within 24 h while Cx40 remained unaffected [79]. The increase in Cx43 was reflected by enhanced GJ conductance in double-cell patch clamp experiments, so that the increase in Cx43 expression may result in a higher number of functional channels [79]. The ET effect was mediated via ET<sub>A</sub> receptors and the AT-II effect via AT<sub>1</sub> receptors. Downstream it was found that the enhanced Cx43 expression was dependent on ERK1/2 (ET) or both ERK1/2 and p38 signal pathway (AT-II).

Thus, ET and AT differentially regulate connexin expression in cardiomyocytes (neonatal rat) with induction of Cx43 whereas Cx40 is not affected. This might be the case in a pathophysiological situation in which similar changes in connexin expression have been observed (i.e. renovascular hypertension [80]) or cardiac hypertrophy.

In addition, bFGF can induce Cx43 expression in cardiac fibroblasts within 6 h after administration [81]. This was associated with enhanced intercellular communication as assessed by scrap load technique. Such regulation of intercellular fibroblast communication might play a role in cardiac scar tissue or might be of importance in arrhythmogenesis in cardiac fibrosis. It has been shown that activation can be conducted even via non-excitable cells like fibroblasts [82]. Interestingly, in cardiomyocytes bFGF exposure acutely (within 30 min) decreased GJ coupling in a PKC $\epsilon$ -dependent mechanism [11].

Among the factors involved in cardiac disease cytokines, TNF $\alpha$  may also play a role in the chronic regulation of connexin expression. In endocardial biopsies from heart transplant recipients, expression of Cx43 was found to be significantly diminished during acute cellular rejection [83]. TNF $\alpha$  downregulated cardiac Cx43 expression in bacterial lipopolysaccharide-induced cardiac inflammation in an in vivo rat model [84]. In contrast, in lung and kidney Cx43 was increased in these experiments [85]. In cultured HeLa-Cx43 cells, however, TNF $\alpha$  (10 U/ml, 24 h) led to increased Cx43 expression in the presence of p38 MAPK [86]. This was also shown in neonatal rat cardiomyocytes [87]. In contrast, in endothelial cells, 0.5 nM TNF $\alpha$  did not affect Cx43 expression but led to downregulation of Cx40 and Cx37 [88], indicating that in different cell types different signaling pathways connected to Cx43 regulation are activated by TNF $\alpha$ . It has further to be taken into account that higher concentrations of

TNF $\alpha$  can also induce apoptosis and cell death, which might also influence gene transcription. Regarding the septic shock model with lipopolysaccharide exposure, many other factors and complex hemodynamic changes may also play a role. However, this inflammation-related alteration in connexin expression may contribute to arrhythmia in patients with inflammatory diseases or septic shock.

Since in the Cx43 promotor region there is an estrogen-responsive element, it has been speculated that this may also be involved in connexin regulation in the cardiovascular system. However, there is only one study in ovariectomized female Wistar rats showing significantly reduced Cx43 in media and endothelium of mesenteric arteries with a concomitant decrease in response mediated by endothelium-derived hyperpolarizing factor [89], which both were normalized by 17 $\beta$ -estradiol supplementation, indicating a possible involvement of estrogen in Cx43 regulation in endothelial and myoendothelial GJs.

In addition to mediators mechanical stretch can also influence connexin expression: cyclical mechanical stretch in neonatal rat cardiomyocytes increased Cx43 while Cx40 expression was not affected [90]. However, the observed increase in Cx43 did not seem to be related to de novo synthesis and thus might be caused by an altered transcript steady state level. Since mechanical stretch can induce the release of AT-II, which according to Polontchouk et al. [79] can induce Cx43 expression, it may be an indirect effect. In accordance with these considerations, stretch-induced Cx43 expression could be inhibited by the AT<sub>1</sub> receptor antagonist losartan [91]. On the other hand, Pimentel et al. [92] also found increased Cx43 expression levels under stretch in association with VEGF secretion and enhanced conduction in cultured neonatal rat cardiomyocytes. The stretch-induced increase in conduction could be antagonized by a VEGF antibody as well as by a transforming growth factor- $\beta$  antibody so that stretch might induce the increase in Cx43 expression via a transforming growth factor- $\beta$ /VEGF pathway [92].

Taken together, these mediators – in concert with mechanical stretch – may contribute to the formation of an arrhythmogenic substrate in cardiac disease by alteration in the Cx43/Cx40 ratio, total connexin expression and regional or subcellular connexin distribution.

Connexins are synthesized in the endoplasmic reticulum, folded and transported to the Golgi network where they are oligomerized to hexameric hemichannels (connexons). These are transported to the plasma membrane [93], and inserted adjacent to, or in conjunction with cadherins and a zonula occludens protein (ZO-1). The intracellular connexin trafficking can be inhibited pharmacologically, e.g. by the metabolic inhibitor monensin [94], disrupting the transport from the Golgi network to the membrane. Connexon assembly (hexamerization) occurring after transport through cis, medial and trans-Golgi

cisternae can be blocked by brefeldin A [95]. The authors concluded that the connexon assembly takes place in the trans-Golgi network in contrast to other integral membrane proteins.

Connexons dock to each other forming the complete GJ channel by interaction of their extracellular loops. Antibodies raised against these extracellular domains have been shown to inhibit GJ assembly. Pharmacologically, it should be possible to interfere with the docking process by adding peptide sequences resembling only the extracellular loops, as first described for Cx32, using them as a kind of competitive inhibitors. Accordingly, using chick cardiomyocytes the motifs QPG and SHVR in extracellular loop 1 and SRPTEK in loop 2 were identified [96]. In rabbit ear arteries, the peptides GAP27 and GAP26 were used as inhibitors of GJIC [97]. A peptide analogue to the second extracellular loop of Cx43, P180–195 (SLSAVYTCKRDPCPHQ; 500  $\mu$ M) can inhibit GJIC via Cx43 in A7r5 smooth muscle cells. Another analogue to the second extracellular loop of Cx40, P177–192 (FLDTLHVCRRSPPHP; 50  $\mu$ M) inhibits coupling via Cx40 channels [98]. Twenty-four-hour treatment of the cultured cells suppressed that portion of intercellular coupling, which could be ascribed to the target connexins by suppression of the unitary conductance specific for the targeted connexin. A peptide homologous to the carboxy terminus was used as control peptide and did not affect coupling. Most often, experimentators use 43GAP27 peptide (SRPTEKTIFII) for inhibition of docking, a peptide which is analogous to the extracellular loop of Cx43. At 300  $\mu$ M, the endothelial component of cannabinoid-induced relaxation is inhibited in rabbit mesenteric artery [47]. Moreover, acetylcholine-induced relaxation in rabbit central ear artery could be antagonized by 300  $\mu$ M GAP27.

Finally, it is possible to interfere with the degradation of connexins. Connexins can be degraded either by the proteasomal pathway or via the lysosomal pathway. Connexins can be degraded via the lysosomal pathway after internalization (so-called annular GJs). Alternatively connexins can be degraded via the endoplasmic-reticulum-associated proteasomal pathway, which seems to be the prominent pathway for misfolded or improperly oligomerized connexins [99]. Degradation of Cx43 seems to be partially dependent on prior ubiquitinylation of the protein. The lysosomal pathway can be inhibited by protease inhibitors such as leupeptin or by disruption of the pH gradient in the lysosome using chloroquine, primaquine or bafilomycin A, leading to enhanced Cx43 presence in the cells [100]. The proteasome can be inhibited by ALLN (acetyl-leucyl-leucyl-norleucinal), lactacystin, clasto-lactacystin and epoxomicin. Interestingly, Laing et al. [100] showed that heat stress led to reduced Cx43 expression, which could be prevented by lactacystin, ALLN and chloroquine. These authors concluded that heat stress may mimic other stress such as ischemia, which also is known to reduce Cx43 expression.

## Summary and Perspectives

The C-terminal tail of several connexins contains a number of serine/threonine and tyrosine phosphorylation sites potentially important for the regulation of connexin functions, and appears to be involved in several steps of the turnover of several connexin types (interfering with oligomerization, transport to the plasma membrane, connexon assembly and disassembly, connexin translocation and degradation by the proteosomal or lysosomal systems). However, other regulatory proteins, behaving as targets for enzymes catalyzing phosphorylation/dephosphorylation, might also be involved in the modulation of the open probability of junctional channels.

The importance of the protein phosphorylation/dephosphorylation balance in the regulation of connexin trafficking, assembly and disassembly, as well as the modulation of the opening state of junctional channels is progressively gaining support. Loss of GJs is a common feature of the failing heart and GJ remodeling is associated with arrhythmic activity in pathological settings, such as myocardial infarction. JNK-induced Cx43 downregulation was for example seen to have significant functional consequences on the electrical propagation in the heart [21]. However, the precise mechanism of GJIC modulation still remains unclear, and future studies will be required to establish the mechanisms by which protein phosphorylation of connexin itself, and plausibly of associated regulatory proteins, regulate the function and processing of connexins throughout their life cycle, and the pathophysiological relationship between changes in phosphorylation and impaired cell-to-cell coupling. Pharmacology provides tools for acute inhibition or promotion of coupling and for influencing synthesis, docking and degradation of connexins. Some of these drugs, as the anti-arrhythmic peptides, were shown to be of therapeutic interest.

## Acknowledgments

Due to space limitations, only a selection of the studies available in the literature on this topic are cited in this study, and we apologize for the curtailed citations and the frequent cross-references to recent reviews.

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## Structural and Functional Coupling of Cardiac Myocytes and Fibroblasts

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### Abstract

Cardiac myocytes and fibroblasts form extensive networks in the heart, with numerous anatomical contacts between cells. Fibroblasts, obligatory components of the extracellular matrix, represent the majority of cells in the normal heart, and their number increases with aging and during disease. The myocyte network, coupled by gap junctions, is generally believed to be electrically isolated from fibroblasts in vivo. In culture, however, the heterogeneous cell types form functional gap junctions, which can provide a substrate for electrical coupling of distant myocytes, interconnected by fibroblasts only. Whether similar behavior occurs in vivo has been the subject of considerable debate. Recent electrophysiological, immunohistochemical, and dye-coupling data confirmed the presence of direct electrical coupling between the two cell types in normal cardiac tissue (sinoatrial node), and it has been suggested that similar interactions may occur in post-infarct scar tissue. Such heterogeneous cell coupling could have major implications on in vivo electrical impulse conduction and the transport of small molecules or ions in both the normal and pathological myocardium. This review illustrates that it would be wrong to adhere to a scenario of functional integration of the heart that does not allow for a potential active contribution of non-myocytes to cardiac electrophysiology, and proposes to focus further research on the relevance of non-myocytes for cardiac structure and function.

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The normal myocardium contains both myocytes and non-myocytes. Myocytes, the core of the contractile machinery of the heart, occupy the majority of the myocardial tissue volume, but account for only one third of the total number of cardiac cells [1]. Non-myocytes, consisting predominantly of cardiac fibroblasts, but also of endothelial and smooth muscle cells, account for the majority of cells in the myocardium.

Some cardiac regions, such as the sinoatrial node, are even richer in fibroblasts than the rest of the heart [2, 3]. Furthermore, fibroblast content increases with development and aging, as well as in a number of pathological conditions [4–8], and may contribute to the development of cardiac pathologies in senescent or diseased hearts.

Not only the content, but also the spatial organization of the two main cardiac cell types shows significant regional differences in the heart. In the ventricle, cardiomyocytes are arranged in sheets (about two to four cells thick), surrounded by a dense connective tissue network, and bridged both by branches of myocytes and fibroblasts [9–11]. In the sinoatrial node, pacemaker cells can be organized in clusters separated by connective tissue, or interspersed with fibroblasts. In all cardiac tissue areas, however, myocytes and fibroblasts are closely interrelated, with numerous anatomical contacts [11, 12].

Electrical communication between cardiomyocytes is via gap junctions [13, 14]. Whether similar communication between fibroblasts and cardiomyocytes occurs has been the subject of considerable debate [15, 16], and was only recently confirmed in native sinoatrial node tissue [17].

Both myocytes and fibroblasts are essential determinants of myocardial structure and function, and contribute to biochemical, mechanical and electrical signaling in the heart [12]. While the role of myocytes in cardiac electrical activity has been studied in great detail, that of fibroblasts is only starting to emerge.

## **Gap Junctions in Normal and Diseased Heart**

Cell coupling in the heart is mediated by gap junctions, specialized channels which directly connect the cytoplasmic compartments of neighboring cells. Gap junctions are involved in many processes, such as growth, cell differentiation, apoptosis, and electrical and metabolic communication. In the heart, gap junctions are generally assumed to electrically couple cardiac myocytes, where they form low-resistance pathways that enable swift conduction of electrical impulses.

In the cardiovascular system, six different gap junctional proteins [called connexins (Cx)] have been identified: Cx37, Cx40, Cx43, Cx45, Cx46 and Cx50 (numbers represent the molecular mass in kilodaltons [18]). Different compartments of the heart express distinct connexin patterns that determine the particular conduction properties of cardiac tissue. Size, abundance and distribution of gap junctions are essential parameters that determine normal impulse propagation in the heart, crucial for a regular rhythm of cardiac contractions. Changes in gap junction expression, distribution and function are

often associated with conduction abnormalities and may promote arrhythmias [19–22].

The general distribution of gap junction proteins in different cardiac tissues has been widely studied. Cx43 is the main connexin in ventricular myocardium of all mammalian species, and is also present in atrial muscle and in the distal His-Purkinje system [23, 24]. Cx40 is abundant in atrial tissue and the conduction system (in particular in the sinoatrial and atrioventricular nodes) of numerous species, while it is absent in the ventricular myocardium [21, 22, 24–31]. Cx40, Cx43 and Cx37 form gap junctions in the aorta and pulmonary artery endothelium [24, 27, 32]. Cx45 is expressed in parts of the conduction system (sinoatrial node, atrioventricular node and bundle of His [30, 33]), but it is largely absent in the rest of the myocardium [34]. Cx50 has been found in the atrioventricular valves of rat heart [35] and Cx37 in the endothelium of the coronary vasculature [32].

There are divergent reports about the types of connexin proteins forming gap junctional channels in the sinoatrial node. Cx45 and Cx40 have been shown to be the main constituents in several species, including the rabbit [28–30, 36]. There are, however, many discrepancies in findings about the presence of Cx43: some reports conclude that Cx43 is absent in the sinoatrial node of most species, including the rabbit [30, 36–39], while others support the presence of Cx43 in the same region [40–42]. These differences may be species specific or linked to technical artifacts (reliability and specificity of antibodies are notorious sources of uncertainty).

Gap junctions undergo a remodeling process during many cardiac diseases [43, 44]. In the ischemic heart, two major abnormalities occur: downregulation of Cx43 in the well-preserved myocardium distant from infarction, and disruption of the normal Cx43 pattern, with dispersion over the myocyte cell surface at the infarct border zone [14, 45–51]. This gap junction remodeling, which occurs rapidly after infarction [52], promotes slowed and non-uniform conduction, which may increase cardiac susceptibility to arrhythmogenesis. In the hypertrophic heart, Cx43 is upregulated during the initial phase of compensatory hypertrophic growth, with a consequent increase in intercellular coupling, but is downregulated in the following chronic phase, causing a reduction in cell coupling and affecting cardiac conduction properties [49, 53–56]. Most investigations on gap junctional remodeling have focused on Cx43, the most abundant connexin in the heart, and only recent studies have extended the analysis to Cx40 and Cx45 [57–59]. Cx43 levels decrease while Cx40 increases in the human ventricle affected by congestive heart failure, where the Cx40 increase may have a compensatory role, improving cardiac activity of the compromised tissue [57]; Cx40 is downregulated and redistributed, with no changes in Cx43

levels, in the right atrium of patients with chronic atrial fibrillation [58], and Cx43 is downregulated and Cx45 is upregulated in the failing human left ventricle [59].

Cardiac gap junctions are conceptually associated with myocytes and assumed to couple homogeneous cells. A few studies have attempted to label muscle cells (using anti-desmin antibodies [36, 39–41]) or sinoatrial nodal cells (using the neurofilament marker or anti- $\alpha$ -smooth muscle actin [38, 39]), but no clear evidence of the localization of connexins between individual myocytes has been shown. The other large cell population in the heart, fibroblasts, has been largely ignored in such studies. Only recently, combined myocyte and fibroblast identification (using cell-type-specific antibodies: anti-myomesin for myocytes and anti-vimentin for fibroblasts), together with gap junction labeling, has provided a more accurate picture of gap junction distribution patterns and cell coupling in native cardiac tissue.

### **Myocyte-Fibroblast Coupling in Cell Culture**

The ability of fibroblasts to contribute to in vitro impulse conduction has been known for almost 40 years. In the late 1960s, Goshima [60] and Goshima and Tonomura [61] reported synchronization of spontaneous cardiac activity in distant cardiac myocytes, interconnected by one or more fibroblasts, illustrating that myocyte-fibroblast heterogeneous electrical interaction occurs, at least in vitro.

The ability of myocytes and fibroblasts to form homogeneous and heterogeneous gap junctions in vitro has been addressed in direct electrophysiological investigations just over 20 years ago. Rook et al. [62, 63] showed that freshly isolated and cultured cardiac myocytes and fibroblasts readily form functional gap junctions, with single channel conductances (at room temperature) of 22 pS between fibroblasts, 43 pS between myocytes, and 29 pS between myocytes and fibroblasts (a value close to the conductance of Cx45 channels). Since cardiac fibroblasts have membrane resistances in the  $G\Omega$  range, the action potential of a myocyte in a heterogeneous cell pair may drive the membrane potential of an electrically coupled neighboring fibroblast in a way that allows the fibroblast to display myocyte-like action potentials, even if the gap junctional coupling conductance is very small [62–64].

Fibroblasts possess a limited arsenal of ion channels [65], including stretch-activated ion channels [66]. Transfected fibroblasts expressing the voltage-sensitive potassium channel Kv1.3 can modify the electrophysiological properties of neonatal rat cardiomyocytes in culture [67], reconfirming that fibroblasts are electrically coupled to myocytes in vitro.



In 2003, Gaudesius et al. [68] extended the concept of cardiac fibroblasts as conductors of electrical excitation from the single cell level to longer distances. With a combination of functional (optical measurements of impulse propagation) and structural (immunohistochemical) studies, they revealed that cardiac fibroblasts were able to synchronize the electrical activity of cultured neonatal rat myocytes across gaps up to 300  $\mu\text{m}$  in length. The molecular substrate for this in vitro coupling seems to be variable. Both Cx43 [62, 63, 69] and Cx45 [68] have been found at points of heterogeneous and homogeneous cell contact in neonatal myocytes and fibroblasts cocultured in monolayers.

This evidence confirms that myocytes and fibroblasts are able to form electrically conducting gap junctional channels, and that fibroblasts actively influence the electrophysiology of myocytes, in vitro.

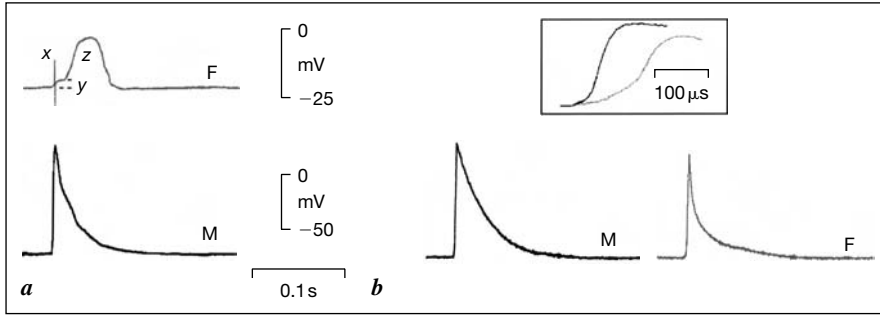
### **Myocyte-Fibroblast Coupling in the Normal Heart**

In vivo, the ability of fibroblasts to directly contribute to cardiac electrophysiology has been proposed since the early 1990s, and has remained the subject of extensive debate [15].

Early electrophysiological studies, conducted using double-barreled microelectrodes inserted into subendocardial layers of spontaneously beating rat right atria, revealed changes in the fibroblast membrane potential that correlated to the action potential in neighboring cardiomyocytes. This provided first circumstantial evidence for myocyte-fibroblast capacitative and electrotonic coupling in vivo (fig. 1) [64].

Functional proof of myocyte-fibroblast coupling in vivo is difficult to obtain by electrophysiological means. A drawback of in vivo electrophysiological studies is that they depend on the electrical identification of cell types (with no visual control), which becomes the more difficult the better a fibroblast is coupled to a myocyte, as the high membrane resistance of fibroblasts causes them to passively ‘mimic’ the electrical activity of the coupled cardiomyocytes (fig. 1b). Electrophysiological studies, therefore, remained inconclusive in the absence of histological proof of cardiac fibroblast-myocyte coupling in vivo.

Initial attempts to define the histological substrate for heterogeneous electrotonic interaction in the heart focused on a tissue that is particularly rich in fibroblasts, even under normal conditions, the sinoatrial node. Transmission electron microscopy studies of serial cross-sections yielded only ‘a single tiny gap-junction-like structure’ between a rabbit sinoatrial node myocyte and a fibroblast in a tissue volume assumed to contain  $10^4$  homogeneous myocyte-myocyte gap junctions, while extended areas of close myocyte-fibroblast



**Fig. 1.** In vivo myocyte-fibroblast electrical interaction. **a** Representative simultaneous recording of the action potential of a cardiomyocyte (M) and the membrane potential of a nearby (40  $\mu\text{m}$  away) fibroblast (F) in the isolated right atrium of rat heart. The fibroblast is weakly coupled to neighboring cardiomyocytes, both capacitatively [initial ‘stimulation artifact’ caused by the action potential upstroke in cardiac myocytes (*x*)] and electrotonically [membrane potential depolarization due to electrotonic interaction, probably via gap junctional channels (*y*)]. The deflection (*z*) is the mechanically induced membrane depolarization, typically observed in cardiac mechanosensitive fibroblasts during tissue contraction; upon electromechanical uncoupling of the tissue, components *x* and *y* remain, while *z* disappears in parallel with mechanical activity. **b** Simultaneous recording of the transmembrane potentials of a cardiomyocyte (M) and a nearby fibroblast (F) that is well coupled to surrounding cardiac myocytes. The action potential in M is representative for those generated by adjacent cardiomyocytes, including any that are in direct contact with F and that drive the electrotonically transmitted ‘mock action potential’ visible in F. The electrotonically induced delay in time to peak is about 100  $\mu\text{s}$  (insert). Modified from Kohl et al. [64], with permission.

membrane appositions were regularly observed [70]. The authors of that study interpreted this as evidence for a lack of heterogeneous cell coupling in native cardiac tissue.

Alternatively, heterogeneous cell coupling could occur via dispersed gap junctional channels in the abundant areas of heterogeneous membrane approximation, potentially forming structures that would not cluster densely enough to provide an electron-microscopically identifiable plaque, as suggested before in pig coronary artery [71].

Extended confocal microscopy studies of living rabbit sinoatrial node and right atrial tissue, involving the vital dye CellTracker<sup>®</sup> (CMFDA 5-chloromethylfluorescein diacetate), reconfirmed the presence of plentiful intimate membrane approximations between myocytes and fibroblasts in this tissue [72]. It has been proposed that these contacts provide a physical site for heterogeneous cell interaction [73], but the possible occurrence of gap junctional channels at these sites was not confirmed until recently.

In 2004, Camelliti et al. [17] used a combination of immunohistochemical and dye transfer coupling techniques, and confirmed the actual presence of functional homogeneous and heterogeneous gap junctions at numerous sites in the rabbit sinoatrial node. Cx40, mainly expressed by non-myocytes, was preferentially found at sites of contact between fibroblasts (fig. 2a, c, d), while Cx45, associated with both myocytes and fibroblasts, was involved in fibroblast, myocyte and myocyte-fibroblast coupling (fig. 2b-d, 3a). Functionality of homogeneous and heterogeneous cell coupling in the rabbit sinoatrial node was confirmed by dye transfer studies using Lucifer yellow (a gap-junction-permeable fluorescent probe), which revealed that fibroblasts form an extensive coupled network of cells able to form conductive bridges between myocytes that are themselves not in direct contact (fig. 3b) [17].

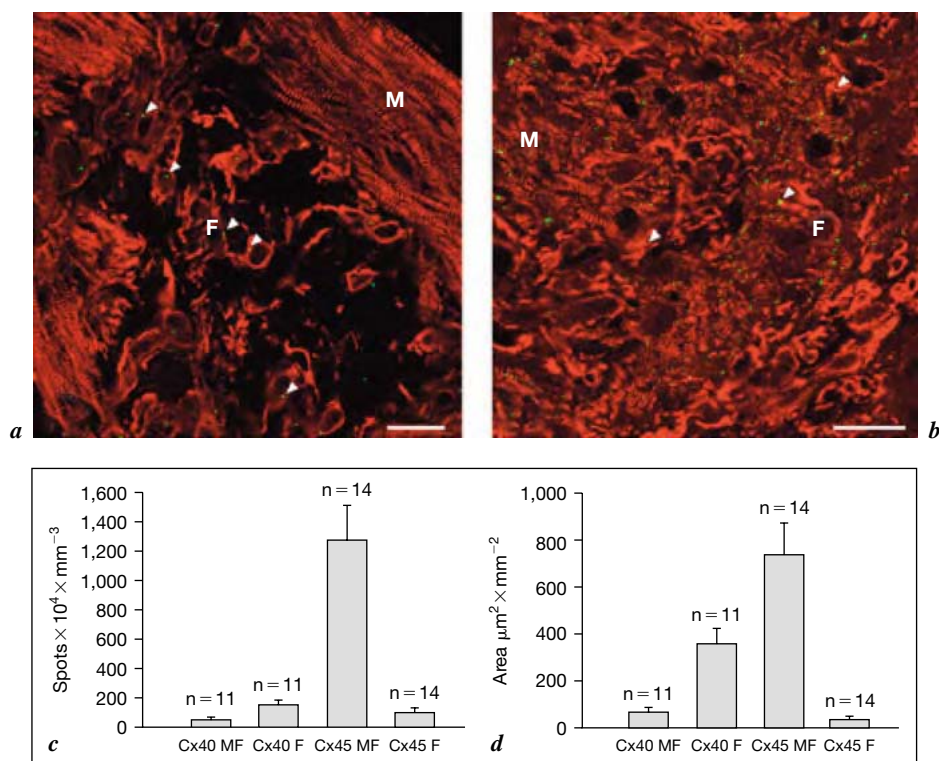
Sinoatrial node fibroblasts express both Cx40 and Cx45, but with a spatially distinct pattern, depending on the histomorphological environment. Fibroblasts in fibroblast-dominated regions devoid of myocytes preferentially express Cx40, which appears to be involved in homogeneous cell coupling. Fibroblasts that are intermingled with myocytes largely express Cx45, which would seem to be able to support both homogeneous and heterogeneous cell contacts (fig. 2). Cx43, Cx32 and Cx50 were absent from rabbit sinoatrial node [17].

While the above study shows myocyte-fibroblast coupling in the sinoatrial node, it is not yet known whether or not similar heterogeneous coupling may also occur in ventricular tissue. Ventricular myocytes and fibroblasts have been very recently shown to express both Cx43 and Cx45 in 4-day neonatal rat hearts [74], with Cx43 localized at points of homogeneous and heterogeneous contacts, and Cx45 mainly confined to fibroblasts and occasionally between the two cell types.

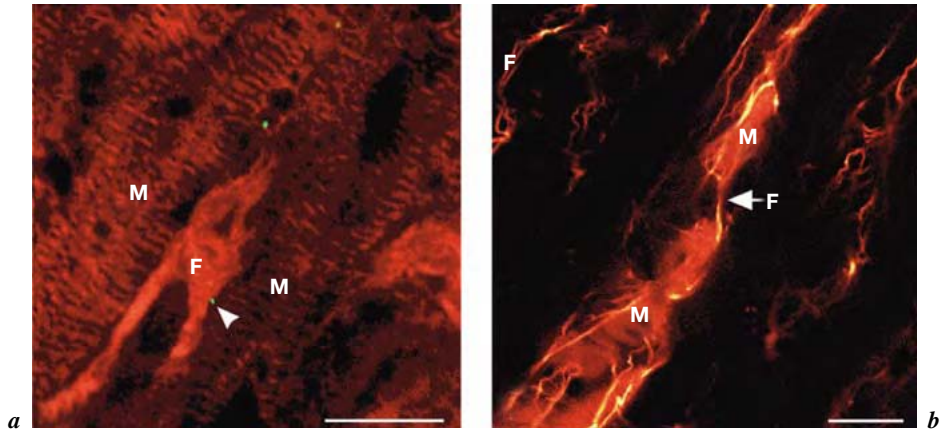
The hypothesis that fibroblasts may be a regular (and regulated) partner to myocyte coupling throughout the whole heart is further supported by preliminary immunohistochemical results showing potential heterogeneous cell coupling in rabbit and sheep ventricular tissue via Cx43 (fig. 4) [75]. Further research is underway to quantify such coupling in the ventricle or other cardiac regions, and to assess its functionality and possible relevance.

### **Myocyte-Fibroblast Coupling in the Diseased Heart**

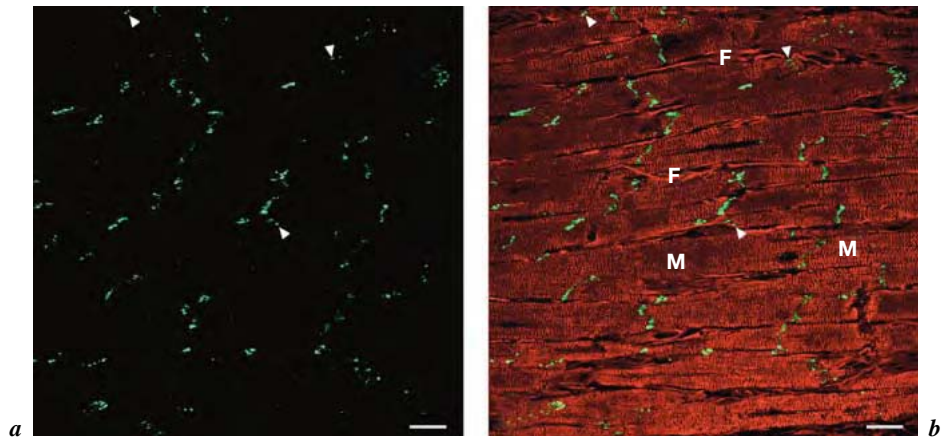
Several pathological states are associated with excessive growth of fibrous tissue, including collagen deposition, fibroblast infiltration and proliferation. Thus, pathological myocardium is often exceptionally rich in fibroblasts, which contribute to altering both structural and functional characteristics of the



**Fig. 2.** Gap junctional expression patterns and levels in rabbit sinoatrial node. **a, b** Confocal images of central sinoatrial node tissue, immunostained with anti-myomesin to mark myocytes (red striated cells, M), anti-vimentin to mark fibroblasts (brightly stained solidly red cells, F) and anti-Cx40 (green dots, **a**) or anti-Cx45 (green dots, **b**). Fibroblasts in fibroblast-rich regions devoid of myocytes express Cx40 (arrowheads in **a**), while fibroblasts that intermingle with myocytes express Cx45 (arrowheads in **b**). Scale bars: 20  $\mu\text{m}$ . **c, d** Quantification of Cx40 and Cx45 density (gap junctions per tissue volume, **c**, and area of gap junction fluorescence per tissue area, **d**) in intermingled myocyte-fibroblast (MF) and fibroblast-rich (F) areas. Cx40 density is significantly greater in F than in MF areas ( $p < 0.001$ ). In contrast, Cx45 density is significantly greater in MF than in F regions ( $p < 0.0001$ ). Both connexins have similar levels, in terms of spots per volume unit, in F areas (Cx40 F vs. Cx45 F in **c**), but in terms of gap junction fluorescence per tissue area, Cx40 is the most abundant connexin associated with fibroblasts (Cx40 F vs. Cx45 F in **d**;  $p < 0.0001$ ). In MF regions, Cx45 is the predominant gap junction, while Cx40 is present at very low levels (Cx45 MF vs. Cx40 MF in **a, b**;  $p < 0.0001$ ).



**Fig. 3.** Myocyte-fibroblast structural and functional coupling in rabbit sinoatrial node. **a** Confocal image showing myocytes (red striated cells, M), fibroblasts (brightly stained solidly red cells, F) and Cx45 (green dots). One of the Cx45 spots (arrowhead) in the given optical plane lies between a myocyte and a fibroblast. **b** Lucifer yellow spread through a heterogeneous chain of fibroblasts (F; thin, brightly labeled cells) and myocytes (M; larger 'orange' cells). Dye-filled myocytes form two groups interconnected only by dye-filled fibroblasts (arrow). Scale bars: 10 (**a**) and 20  $\mu\text{m}$  (**b**). From Camelliti et al. [17], with permission.



**Fig. 4.** Cx43 in sheep normal ventricular tissue. **a** Single labeling for Cx43 (green dots) shows intercalated disks, but also some dispersed punctate labeling (arrowheads), which would – in the absence of cell type identification, potentially be discarded as 'noise'. **b** Triple labeling for Cx43 (green), myocytes (striated red, M) and fibroblasts (red, F) reveals Cx43 not only in intercalated disks between myocytes (as expected), but also at points of contact between myocytes and fibroblasts (arrowheads). Scale bars: 20  $\mu\text{m}$ . From Camelliti et al. [75], with permission.

diseased heart. Fibroblasts, coupled to other fibroblasts and/or to myocytes, may play an important role in the biochemical, mechanical and electrical behavior of the pathological heart and in the structural and functional remodeling.

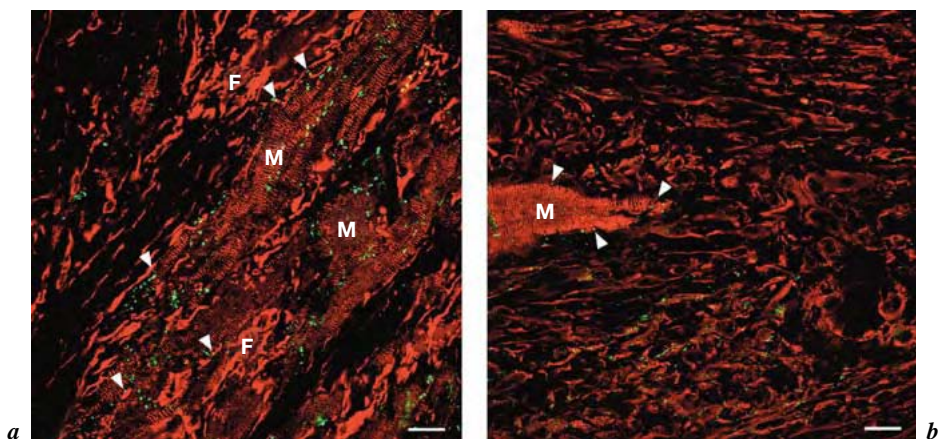
A recent report highlighted extensive changes in gap junction distribution and cell coupling in a sheep coronary occlusion infarct model (from 12 h to 4 weeks after infarction [75]). Cx43, which is normally organized in intercalated disks between myocytes, undergoes dramatic remodeling after myocardial infarction, acquiring a punctate dispersed pattern along the surface of structurally compromised myocytes that intermingle with fibroblasts in the so-called 'infarct border zone'.

Fibroblasts in sheep infarcts (in contrast to previous observations in human and rat scar tissue [45, 76]) express Cx45 and/or Cx43, but not Cx40. They form gap junctions with fibroblasts and, possibly, other cells in the infarct [75]. Two spatially and temporally distinct fibroblast phenotypes were identified based on connexin patterns. Cx45-expressing fibroblasts appear in the damaged tissue within a few hours after infarction and reach their peak density after 1 week, while Cx43-expressing fibroblasts emerge later and continue to increase in number until at least 4 weeks after infarction [75]. Cx43- and Cx45-expressing fibroblasts were systematically found in close proximity to myocytes at the infarct border zone (fig. 5a). Further investigation is needed to assess functionality of homo- and (potentially) heterogeneous coupling, to quantify contacts, and to establish their role in the remodeling process after infarction.

Of note, islands of morphologically normal myocytes were regularly found inside the scar tissue, surrounded by tissue with high fibroblast density. Some of these myocyte islands were not detectably bridged via strands of myocytes to the healthy 'bulk' myocardium [75]. These viable myocytes form close contacts with the surrounding Cx43-expressing fibroblasts (fig. 5b), and it will be interesting to investigate whether and how they are functionally integrated with the normal myocardium.

One possible scenario would involve fibroblast-based connections. This concept is supported by the recent discovery of electrical impulse conduction from rabbit bulk myocardium into transmural scars 8 weeks after myocardial infarction. Interestingly, optically recorded epicardial action potential waveforms inside the scar region were similar in shape to cardiac action potentials, except for their somewhat reduced amplitude and upstroke velocity [77, 78]. In this regard, they were very similar to the intracellularly recorded activity of cardiac fibroblasts that are well coupled to a myocyte source, as seen in vivo (fig. 1b) [64] and in vitro [63].

The optically recorded action potentials in the scar could originate either from the remaining dispersed cardiomyocyte islands in the fibrous tissue (but this would require fibroblasts to act as conduction lines for their excitation), or



**Fig. 5.** Gap junction distribution and cell coupling in ventricular infarcts. Confocal microscopy sections of sheep ventricular infarct border zone (*a*) and mid-infarct scar tissue (*b*), immunostained with anti-myomesin to mark myocytes (red striated cells, M), anti-vimentin to mark fibroblasts (solidly red cells, F) and anti-Cx43 (green dots). Cx43 is expressed both by fibroblasts and myocytes, and is present at the interface between the two cell types (arrowheads), where it could potentially be involved in myocyte-fibroblast coupling. Scale bars: 20  $\mu$ m.

directly from cardiac fibroblasts (whose transmembrane potential may mimic cardiac action potentials). Both the fact that excitation spreads into the scar tissue and that the observed waveforms resemble those of cardiomyocytes (in the absence of significant numbers of surviving cardiomyocytes) are compatible with the notion that fibroblasts contribute to impulse conduction in this particular experimental model [79].

### Relevance of Myocyte-Fibroblast Coupling

The structural, biochemical and mechanical relevance of myocyte-fibroblast interrelation in normal and diseased heart has been firmly established. The two cell types have been shown to be able to regulate cardiac responses to mechanical stimulation and pathological conditions, building on cross-talk that involves mechanical interaction and humoral factors released by the two cell populations [8, 80].

In the normal and diseased myocardium, fibroblasts form a highly coupled network [17, 75] that, at least in the sinoatrial node, is functionally linked to myocytes. Fibroblasts may, therefore, contribute to several chemical and biochemical processes, such as metabolite regulation, nutrient transfer, waste

removal and biochemical signaling, similar perhaps to astrocytes in the nervous system [81, 82]. In addition, the highly coupled fibroblast network in the diseased myocardium could also be involved in inflammatory responses, lesion spread, and myocardial remodeling, including regulation of cell proliferation and migration, extracellular matrix remodeling, and release of cytokines and growth factors after myocardial infarction [83, 84]. In diseased hearts, direct fibroblast-fibroblast and fibroblast-myocyte coupling could provide a substrate for progressive infarction via gap-junction-mediated bystander effects, where fibroblasts contribute to the spread of remodeling-related signals from the infarcted myocardium to surrounding tissue via gap junctions, potentially similar to the spread of neuronal damage via coupled astrocytes [12, 85, 86].

The relevance of the myocyte-fibroblast interrelation in cardiac electrical function has been, and still is, a controversial subject. This is caused, in part at least, by the difficulty in extrapolating from structural data and/or circumstantial evidence to actual functional relevance (thus far, functionality of coupling between cardiac fibroblasts and myocytes has been firmly established for rabbit sinoatrial node only). In addition, it is not always easy to confidently identify fibroblasts on the basis of morphological parameters (most labels are not strictly specific) or in situ cell electrophysiology (fibroblasts mimicking transmembrane potential dynamics of coupled heterotypic neighbors), and even the very concept of what a fibroblast actually represents is not uniquely defined: a specific cell type; a pluripotent cell; a transitory stage of cell development; or anything we cannot place into any other category?

Generally, fibroblasts have been considered to affect cardiac electrophysiology only passively. By forming electrically insulating septa between groups of muscle cells, in particular in aged and diseased sinoatrial nodes, or in areas of fibrosis and scarring, they act as passive obstacles for electrical excitation, contributing to discontinuous conduction, increased cardiac electrical heterogeneity and arrhythmogenesis [87, 88].

Both in vitro [63, 68] and in vivo evidence [17, 64] suggest that cardiac fibroblasts can be structurally and functionally coupled to other fibroblasts and myocytes via gap junctions. Such coupling would allow fibroblasts in vivo to influence cardiac electrical activity not only passively but also actively, by modulating electrical properties of cardiac tissue or by bridging gaps between excitable cells. The potential role of such electrical coupling varies in normal and diseased heart, in different cardiac tissues, and with age, and it could be particularly relevant in regions with high fibroblast content, like the sinoatrial node or infarct tissue.

Fibroblasts coupled to myocytes could act as current sink. By imposing an electrical load, they could induce regional shortening of action potential duration, arrhythmogenic excitability gradients, electrotonic depression of the tissue, and



contribute to slow conduction and unidirectional block, in particular in ischemic zones or, for example, in the atrioventricular node, where fibroblast content is exceptionally high.

Alternatively, fibroblasts coupled to myocytes could be involved in impulse conduction over short or long distances. As short-distance conductors, fibroblasts may interconnect groups of sinoatrial node cells separated by fibroblast aggregations, or layers of myocardial tissue separated by connective tissue and/or extracellular gaps in the ventricular wall. Here fibroblasts could provide electrical coupling that supports synchronization of the heterogeneous sinoatrial node, or contributes to the smoothing of electrical propagation in the cross-sheet direction of healthy ventricular tissue. As long-distance conductors, fibroblasts could bridge donor and recipient tissue after heart transplantation (in 10% of cases, such donor-recipient coupling manifests itself in patients [89]), or connect islands of surviving myocytes, found inside scar tissue, with the surrounding healthy myocardium. Here fibroblasts, via gap junctional coupling with other fibroblasts and with myocytes, could be responsible for the electrical integration of post-transplantation tissue across connective tissue barriers, for the impulse invasion of ventricular infarct scars, and conduction of electrical activity across the scar tissue. Such fibroblast bridges could have beneficial or detrimental effects. On one hand, they could provide a mechanism for electrical communication and synchronization of excitable tissue separated by non-excitable barriers, but on the other hand, the slow conduction, which occurs across long fibroblast inserts [68], could be arrhythmogenic.

Potential therapeutic treatments could involve changes in electrical coupling between myocytes and fibroblasts. It is by no means simple to predict whether an increase or reduction in coupling should be favored, as both might facilitate normal electrical propagation, or induce arrhythmia. Selective enhancement of myocyte-fibroblast and fibroblast-fibroblast coupling during cardiovascular diseases (produced, for example, via cardio-specific fibroblast connexin over-expression) would increase metabolic and electrical coupling across fibrotic or scar tissue and potentially avoid or repair cardiac conduction defects (such as fragmented or slow conduction, block, or sick sinus syndrome) in order to prevent arrhythmias [90]. On the other hand, uncoupling may be the preferred intervention, since this has been shown to limit necrosis and infarct size in ischemic hearts, probably by reducing lesion spread via gap-junction-mediated bystander effects [91, 92]. Reducing connexin expression after myocardial infarction, ideally targeted at cardiac fibroblasts, might limit cell-cell-mediated propagation of damage and thus reduce the extent of cardiac dysfunction, and preserve passive and active properties of the surviving myocardium.

Furthermore, fibroblasts are mechanosensitive: they respond to mechanical stimuli, such as those imposed by the contractile activity of the surrounding

myocardium, with changes in their membrane resistance and potential (component  $z$  in fig. 1a) [93]. By direct gap junctional coupling with other fibroblasts and/or adjacent myocytes, they could be involved in heart rate response to changes in the mechanical environment [72], or in the local depolarization and triggering of action potentials in connected myocytes, observed in cardiac scar models subjected to transient stretch [94].

In conclusion, the dogma of an electrically coupled network of cardiomyocytes, which is not connected to other cell types in the heart, can no longer be sustained. The extent, regulation and role of heterogeneous cell coupling in normal and diseased heart require further targeted research. This research needs to contain a strong element of *in situ* work, since *in vitro* gap junction expression and coupling differ significantly from (patho)physiological conditions. Projecting further ahead, therapeutic strategies that target fibroblasts and/or myocyte-fibroblast coupling could become an interesting avenue for medical intervention and, possibly, treatment of arrhythmias; the pharmacological and genetic tools for amending fibroblast proliferation and coupling have started to emerge [95, 96].

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## Connexins and Cardiac Arrhythmias

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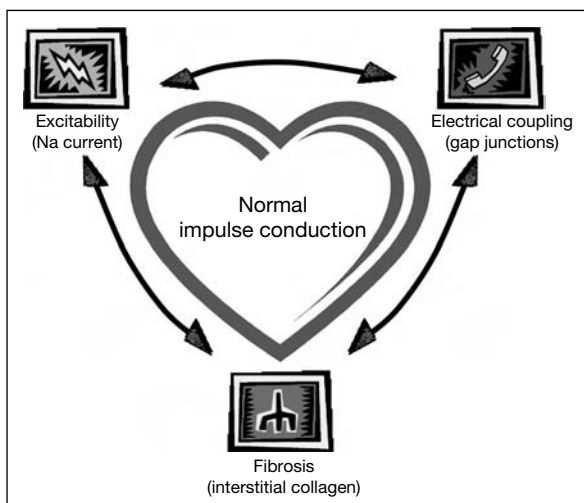
### Abstract

During cardiac remodeling, impulse conduction in the heart is altered by changes in excitability, electrical coupling, and tissue architecture. The impairment of normal impulse conduction is one of the factors that increases the propensity for arrhythmias. This chapter focuses on the relationship between electrical coupling between ventricular myocytes and arrhythmogenesis. Mouse models of decreased electrical coupling in the heart have shown that a clinically relevant 50% reduction in gap junctions in the heart has no effect on impulse conduction or arrhythmogenesis. To impair conduction and arrhythmias, coupling has to be reduced to very low levels. Apparently, there is a large conduction reserve, which can preserve normal impulse conduction even when electrical coupling is moderately reduced. However, cardiac remodeling is also associated with reduced excitability and increased levels of collagen deposition (fibrosis). It is therefore presumably the combination of, in itself ineffective, reduction of electrical coupling with other impairments like fibrosis or reduced excitability that causes the limits of conduction reserve to be exceeded, thereby resulting in abnormal impulse conduction and enhanced arrhythmogenesis.

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Cardiac remodeling is an adaptive process in response to increased workload in order to preserve cardiac output. Increased workload may result from multiple factors, such as coronary artery disease, myocardial infarction, hypertension, valvular diseases, cardiomyopathy, congenital heart disease, lung disease, diabetes, anemia, hyperthyroidism, or arrhythmia/dysrhythmia. This remodeling process includes cellular hypertrophy and functional changes which



**Fig. 1.** Determinants of impulse propagation.

may lead to heart failure in the course of time. During this remodeling process, the major parameters of cardiac impulse propagation are affected. Normal propagation of the electrical impulse through the heart requires low-resistance cell-to-cell coupling, proper excitability of the cardiac myocytes, and normal tissue architecture (fig. 1). The electrical coupling between myocytes is mediated by gap junctions. In the ventricular working myocardium connexin43 (Cx43) is the main gap junction protein between myocytes [1]. The excitability of the cardiac myocytes is largely determined by their fast sodium current, with the *SCN5A* gene encoding the pore-forming ion channel proteins. Myocytes are embedded in a network of collagen fibers which provides the physical framework for transmission of force through the myocardium. In the normal heart, the extracellular matrix consists of thin intertwining strands, allowing cell-to-cell contact between neighboring cardiac myocytes.

In patients with ventricular remodeling, it has been reported that: (1) electrical coupling is reduced by a 30–50% decreased Cx43 expression [2–7], (2) excitability is reduced by reduction of sodium channel expression up to 50% [8, 9], and (3) intercellular collagen deposition (fibrosis) is increased from 5 to 30% [10–12]. These changes increase the arrhythmogenic vulnerability of the heart which is reflected by the high sudden death rate in patients with congestive heart failure [13, 14].

Clearly, the onset, progress and arrhythmogenic causes and consequences of electrical and structural remodeling are very diverse. This chapter will focus



on the relationship between changes in ventricular gap junction expression, impulse conduction, and arrhythmias, in order to evaluate the contribution of reduced Cx43 expression to the arrhythmogenic substrate.

### **Electrical Uncoupling and Arrhythmogenesis**

According to Coumel [15], 3 factors determine the propensity for arrhythmias. (1) the arrhythmogenic substrate, (2) initiating triggers, and (3) modulating factors. Reduced levels of coupling mainly increase the arrhythmogenicity of the substrate by promoting/facilitating reentry, although reduced levels of coupling are also associated with focal activity [16, 17].

Conduction velocity (CV) and shortening of the effective refractory period (ERP) are major determinants of arrhythmogenicity. Their combined effect is described by the wavelength ( $\lambda$ ) which is defined as [18]:

$$\lambda = CV * ERP \quad (1)$$

A reduction of CV and/or ERP will decrease  $\lambda$ . A short wavelength increases the likelihood that one or multiple reentrant circuits can be accommodated by the heart. Even hearts as small as that of a mouse can sustain fibrillatory activity, given the right circumstances [19, 20]. Besides a reduction in  $\lambda$ , local dispersion may be significant as well. Disproportional changes in the conduction velocity parallel ( $CV_{\text{long}}$ ) or perpendicular ( $CV_{\text{trans}}$ ) to the fiber orientation alter the anisotropic ratio, and may affect the propensity for anisotropic reentry [21] as well as focal activity [17]. The inhomogeneity of the substrate is also a major determinant of arrhythmogenesis. Dispersion in conduction velocity was found to favor reentrant arrhythmias [22]. A second important factor is dispersion in ERP [23].

Furthermore, uncoupling may have paradoxical effects on safety of conduction [24, 25]. The safety factor describes the relationship between current source and current sink. During propagation, the excited cells act as current source to depolarize the quiescent downstream cells (current sink) towards their threshold. If the amount of source current exactly matches the required sink current, safety factor (SF) is 1. A  $SF > 1$  means that more current is generated during excitation than needed for exciting the sink cells. If  $SF < 1$  conduction block occurs. Computer modeling and in vitro studies showed that mild uncoupling increases SF, and can sustain very slow conduction successfully; however, a high level of uncoupling reduces SF and results in conduction block [24, 26]. Regions with low SF are sensitive to premature activation, as it will result in reduced current availability at the source, because the fast sodium channels are not fully recovered from inactivation at the time of premature activation. This

may further reduce SF to levels  $<1$  causing (unidirectional) conduction block. In general, global uniform uncoupling preferentially leads to conduction block in the transverse direction [20, 27, 28].

## Ventricular Conduction Velocity and Arrhythmias

### *Mouse Models of Decreased Ventricular Coupling*

Cx43 is the main gap junction protein between myocytes of the ventricular working myocardium [1]. The role of Cx43 cannot, however, be determined in mice homozygously deficient for Cx43 because they die perinatally, due to a cardiac malformation at the pulmonary outflow tract [29–31]. Mice heterozygously deficient for Cx43 showed reduced expression of total Cx43 to levels of  $\sim 50\%$ , also resulting in a comparable reduction of Cx43-based gap junctions [32]. In some studies, this reduction in Cx43 expression was associated with increased activation delay of the ventricles and reduced conduction velocity [33–36]. However, other studies have shown that a similar 50% reduction of Cx43 expression did not alter conduction velocity in the mouse heart [20, 37].

To circumvent the problem of perinatal death, two mouse models were generated independently with conditional deletion of the Cx43 gene, based on the Cre/LoxP system [38]. In one model, the Cre enzyme was under the control of the  $\alpha$ -myosin heavy chain ( $\alpha$ MHC) promoter [39], resulting in deletion of the floxed Cx43 gene ( $\alpha$ MHC-Cre/Cx43<sup>fl/fl</sup>). In the second model, one coding region of the Cx43 gene was replaced by Cre-ER(T), a fusion construct of the Cre recombinase and a specifically mutated version of the ligand binding domain of the human estrogen receptor [40]; the second Cx43 region was floxed and deletion was induced by injection of 4-hydroxytamoxifen (4-OHT; Cx43<sup>CreER(T)/fl</sup> model) [41].

The basic difference between the two models is that in  $\alpha$ MHC-Cre/Cx43<sup>fl/fl</sup> mice, the deletion of Cx43 is determined by developmental parameters, i.e. the time point of  $\alpha$ MHC expression.  $\alpha$ MHC expression is found at embryonic day 9.5, which is before the onset of Cx43 expression (embryonic day 10.5) [42–44], while in the Cx43<sup>CreER(T)/fl</sup> model, the mice express 50% Cx43 until a defined time point at which the animal is injected with 4-OHT, initiating the deletion of the Cx43 gene. The  $\alpha$ MHC-Cre/Cx43<sup>fl/fl</sup> mice die between 1 and 2 months after birth due to fatal arrhythmias [39]. In the Cx43<sup>CreER(T)/fl</sup> model, sudden arrhythmogenic death occurs within 1 month after induced deletion [41].

Detailed mapping was performed in both mouse models. Optical mapping of  $\alpha$ MHC-Cre/Cx43<sup>fl/fl</sup> mouse hearts revealed that left ventricular conduction velocity was significantly slowed up to 55% in the transverse direction ( $CV_{trans}$ ) and 42% in the longitudinal direction ( $CV_{long}$ ), resulting in a significant

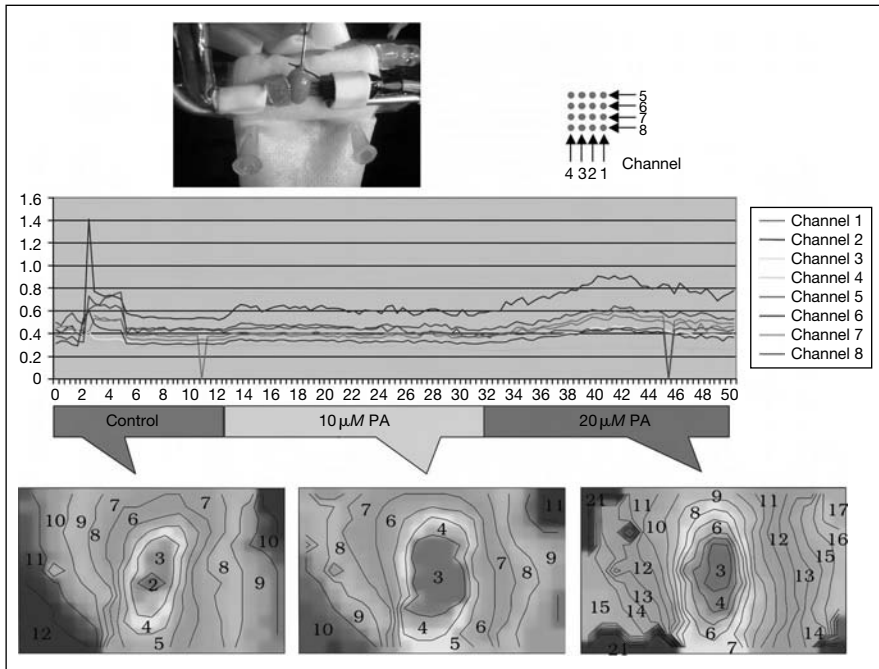
increase in anisotropic ratio compared with control littermates (from 1.7 to 2.1). These reductions in conduction velocity were associated with a 95% reduction in Cx43 expression. The animals developed ventricular arrhythmias, but the underlying mechanism was unclear [39]. The Cx43<sup>CreER(T)/fl</sup> mouse hearts were Langendorff perfused and extracellular electrograms were recorded with a 247-point multi-terminal electrode [20]. Both left ventricle (LV) and right ventricle (RV) were mapped during basic and premature stimulation from the center of the grid. This study showed that a 50% reduction of Cx43 expression does not have an effect on conduction velocity or arrhythmogenesis. However, a 90% decrease of Cx43 protein expression (4-OHT treated Cx43<sup>Cre-ER(T)/fl</sup> mice) resulted in a significant reduction of both  $CV_{Long}$  and  $CV_{Trans}$  in RV and of  $CV_{Trans}$  in LV. The anisotropic ratio increased significantly in both RV and LV. Arrhythmias were exclusively observed in the group with only 10% Cx43. Arrhythmias originated from the RV, showing stable anisotropic reentry and fibrillatory conduction on the LV.

In another study we assessed the effect of pharmacological uncoupling of the myocardium on conduction velocity, using palmitoleic acid (PA), a selective inhibitor of gap-junction-mediated intercellular coupling [45]. RV epicardial mapping was performed on a Langendorff-perfused mouse heart, with simultaneous recording of LV tissue impedance (4-point method [46]; fig. 2). After control measurements, 10  $\mu M$  PA was added to the perfusate, which increased tissue impedance, but did not change conduction velocity. When the PA concentration was increased to 20  $\mu M$ , tissue impedance further increased, now giving rise to conduction slowing, with more pronounced effects on  $CV_{trans}$  than on  $CV_{long}$ .

Only at 20  $\mu M$  PA, did the hearts show enhanced vulnerability to arrhythmias, as shown in figure 3. In this example, one premature stimulus (S1–S2 = 50 ms) resulted in a short run of nonsustained monomorphic VT. It is clear, however, that this short VT results from anisotropic reentry with normal conduction parallel to the fiber orientation (fiber orientation indicated by the blue double-headed arrow), and slow conduction parallel to the fiber orientation. Our results, with increased anisotropy due to a more pronounced reduction in  $CV_{trans}$  than  $CV_{long}$ , compare well with previous reports on pharmacological uncoupling of the myocardium [27, 28, 47–49].

### **Cx43, Electrical Coupling and Conduction Reserve**

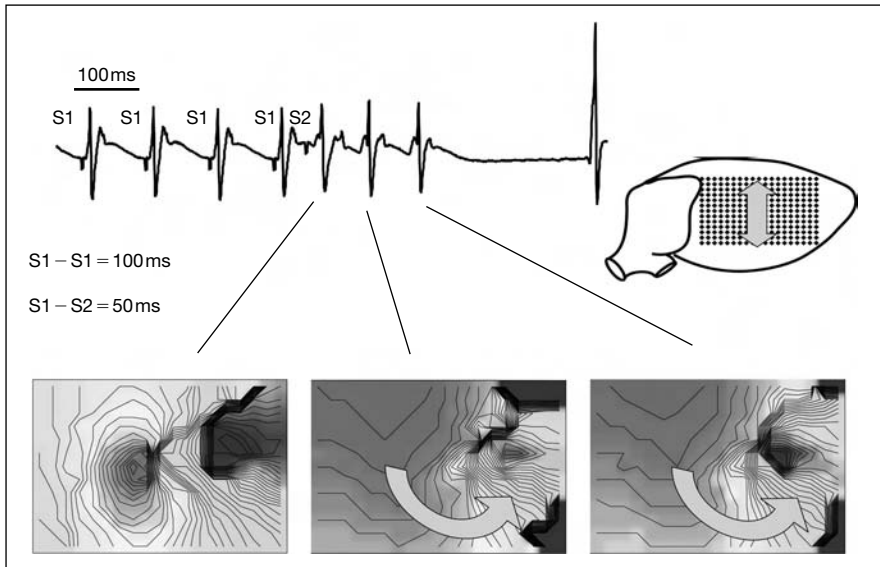
From the Cx43 conditional knockout mouse studies, it is clear that a strong reduction in Cx43 expression slows conduction and enhances arrhythmogenesis. However, as mentioned previously, there is conflicting evidence on mice expressing only ~50% Cx43 as some investigators report a reduced CV [33–36], while



**Fig. 2.** Simultaneous measurement of RV epicardial activation and LV tissue impedance in 8 channels, using the 4-point method [46] in a Langendorff perfused mouse heart. PA, 10 or 20  $\mu M$ , was added to the perfusate. Addition of 10  $\mu M$  PA increases tissue impedance, but does not affect conduction velocity or anisotropy. Further increase of impedance by 20  $\mu M$  PA reduces conduction velocity and increases anisotropy, as demonstrated by the crowding and elongation of isochrones, respectively. Numbers in bottom panels indicate activation times relative to stimulus.

others do not [20, 37]. Interestingly, a strong reduction of Cx43 expression (90% or more) only reduces CV to about half. This nonlinear relationship between electrical coupling and conduction velocity was established by computer modeling studies of action potential propagation in a one-dimensional strand of ventricular myocytes, using the phase-2 Luo-Rudy guinea-pig ventricular cell model [24] or the Priebe-Beuckelmann human ventricular cell model [50]. Figure 4 shows results obtained with linear strands of Priebe-Beuckelmann model cells.

The relation between conduction velocity and gap junctional coupling conductance reveals a flat curve for values of gap junctional coupling conductance in the range of 3–12  $\mu S$  (normal value about 6  $\mu S$  [50]). In the normal heart, the intracellular (myoplasmic) resistance exceeds that of the gap junction resistance and therefore the gap junctions only play a minor role in total resistance.

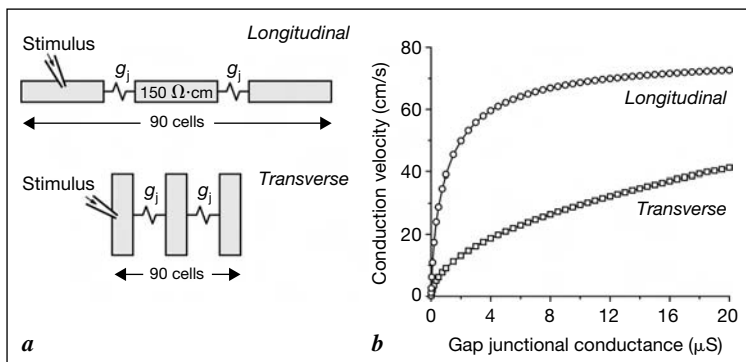


**Fig. 3.** Nonsustained ventricular monomorphic VT after premature stimulation at 20  $\mu\text{M}$  PA. The premature stimulus S2 causes conduction block to the apex, starting 2 reentrant beats. Note the anisotropic nature of the reentrant circuit (arrows), with high conduction velocity (isochrones widely spaced) parallel to the fiber orientation (indicated by the double headed arrow in the heart cartoon), and slow conduction perpendicular to fiber orientation (crowded isochrones).

As such, there is a large ‘conduction reserve’ with little effect of reduced coupling until gap junctional coupling conductance approaches values close to uncoupling ( $<3 \mu\text{S}$ ), at which conduction velocity is significantly reduced [24, 50]. The discrepancy in the literature on the effect of a 50% decrease in Cx43 expression on CV might be explained by the fact that a 50% reduction is near the cut-off point of the steep portion of the CV vs. coupling conductance relationship. Depending on the coupling levels in the normal heart and experimental circumstances, either a reduced CV or no effect can be observed.

### Uncoupling and Ventricular Effective Refractory Period

Mouse models of reduced electrical coupling showed that the average ERP was unchanged when coupling was reduced by half [35, 36] or down to 10% [20]. In rabbit hearts, pharmacological uncoupling showed similar results with regard to the absolute value of activation-recovery intervals, but also showed a clear increase in their dispersion [49]. In comparable experiments on mice we have



**Fig. 4.** Relation between myocardial conduction velocity and gap junctional conductance as determined by computer simulation. **a** Diagram of linear strand model. Action potential conduction is studied in a linear strand composed of 90 human ventricular myocytes [54] that are either arranged end to end (top) or side by side (bottom) and coupled by gap junctional conductance  $g_j$ . Myoplasmic resistivity is set to  $150 \Omega \cdot \text{cm}$ . Action potential propagation is initiated by applying a 1-Hz external stimulus to the leftmost cell of the strand. **b** Longitudinal and transverse conduction velocity vs. gap junctional conductance.

determined the ERP at 9 electrode terminals (1.5 mm apart) of the 247-point multi-electrode. As in the study by Dhein et al. [49], we electrically uncoupled the heart using the specific gap junction blocker PA. Serial infusion of control buffer, and buffers with  $10 \mu\text{M}$  PA or  $20 \mu\text{M}$  PA resulted in a significant increase of the average ERP from 52 to 60 and 63 ms ( $n = 4$ ), while the maximum (absolute) ERP dispersion significantly increased from 38 to 44 and 53 ms, respectively. The reduction of intercellular electrical coupling increases ERP dispersion, presumably due to the unmasking of intercellular differences in action potential duration. These findings compare well to those reported in computer modeling studies [51, 52]. Interestingly, the increase in average ERP due to uncoupling was also reported in a computer modeling study [52]. Strands of either Priebe-Beuckelmann model cells [52] or Luo-Rudy model cells [52, 53] both exhibited prolongation of the action potential duration at reduced intercellular coupling. Apparently, reduction of the load of cells still at resting membrane potential on excited cells by electrical uncoupling prolongs action potential duration.

## Concluding Remarks

In general, it can be concluded that the 30–50% reduction in Cx43 expression that is reported in heart disease [2–7], is by itself not sufficient to reduce

conduction velocity and increase arrhythmogenesis. Very large changes of electrical coupling are required to impair conduction. In heart disease, it is presumably the combination with other factors, such as fibrosis or reduced excitability, that causes the limits of conduction reserve to be approached or exceeded, resulting in slow conduction or conduction block. However, until now, very little is known about the limits of conduction reserve.

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## Role of Connexins in Atrial Fibrillation

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### Abstract

Atrial fibrillation (AF) is the most common arrhythmia in humans. AF is accompanied by a remodeling process which changes the electrophysiology of the cells and the gap junctional communication within the tissue. Gap junctions, forming communicating channels between neighboring cells, and their specific geometric arrangement seem to contribute to the initiation of AF within the pulmonary veins as well as to the stabilization of AF providing a heterogeneous biophysical network of cells enabling multiple wavelets. These tissue changes are accompanied by fibrosis and changes in the expression levels of Cx43 and Cx40, probably depending on the underlying diseases or the animal model used. New studies point to a modulating role of angiotensin II in this process and a possible therapeutic role for ACE inhibitors or AT<sub>1</sub> antagonists.

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Atrial fibrillation (AF) is encountered in 1.5% of the population and is the most common arrhythmia in man [1]. Of people older than 65 years even >5% are affected [2]. The prevalence of AF is also influenced by underlying cardiac diseases such as congestive heart failure, valvular disease, myocardial infarction or ischemic heart disease. The development of AF is associated with an increased risk of death (2.7-fold in women, 1.6-fold in men) [3] and stroke [4]. Clinical experience shows that with prolonged duration of AF it becomes increasingly difficult to convert patients to sinus rhythm and to maintain sinus rhythm after successful conversion.

Regarding the pathophysiology of AF, we have to discriminate between factors involved in the *initiation* of AF and those contributing to *stabilization* and progression of AF. The latter includes atrial, structural, contractile and electrical remodeling.

## Initiation of Atrial Fibrillation

Initiation of AF seems to be associated with age, and age-dependent increased fibrosis and widening of intercellular spaces, which facilitate transversal conduction block as was seen in dog model [5]; it also appears to be associated with atrial stretch following by enhanced atrial volume and pressure, which is often caused by congestive heart failure and enhances dispersion of refractoriness [6]. Enhanced atrial stretch may then activate stretch-mediated channels and may be involved in increases in the  $\text{Na}^+/\text{Ca}^{2+}$  exchanger thus altering  $\text{Ca}^{2+}$  and  $\text{Na}^+$  homeostasis and indirectly modulating action potential duration [7]. Atrial stretch is associated with slowing of conduction and increased dispersion of refractoriness. Due to the special architecture, a critical situation may result in the area of the transition from the atrium to the pulmonary veins [8]: in most cases (85–95%) sleeves protruding from the atrium into the veins are found there. Interestingly, these sleeves form circular or semi-circular electrically coupled structures, the coupling being realized by Cx43 and to a lower extent Cx40, as was elegantly shown in canine pulmonary veins [9]. This circumferential pattern has been postulated by these authors to provide a substrate for rapid circular reentry which in most cases might form the primary source (rotor) of AF. Accordingly, the initiation of AF has been shown to originate from the pulmonary veins in patients by Haissaguerre et al. [10]. Other factors suggested to be involved in the initiation of AF are increased atrial natriuretic factor (ANF) [11] which can cause the deposition of ANF-dependent amyloid [12–14], atrial ischemia, or changes in the autonomic nervous system as well as increased release of angiotensin [for a review, see ref. 8]. These studies have in common to postulate a structural factor (circumferential pattern of coupling in the veins), heterogeneities (by fibrosis, amyloid deposition, stretch, ischemic scars) and regulatory factors (angiotensin II, ANF,  $\text{Ca}^{2+}$  influx, etc.). From the literature, however, it becomes evident that a certain geometric pattern in the electrical impulses (or architecture) to the tissue appears to be very important and provides the biophysical basis for AF. AF has been considered as a multiple wavelet re-entrant activation of the atria [15], probably with an – initial – reverberator located near the pulmonary veins [10]. To allow and maintain a multiple wave re-entry, an electrical network has to be provided which enables such circus movements. This seems to be realized in AF by a certain pattern of intercellular gap junctions coupling cells and other factors (e.g. fibrosis or amyloid depositions) separating other cells. In a goat model of pacing-induced AF, it was shown that in the beginning the waves are more homogeneous broad fibrillation waves while after remodeling, i.e. after >2 weeks, the fibrillation waves become much more disorganized and complex [11, 16–17]. This increasing complexity has been considered to indicate a structural change of electrical

networking and a change in the atrial ionic channels. This remodeling is assumed to participate in maintenance of AF.

### **Stabilization of Atrial Fibrillation**

‘Atrial fibrillation begets atrial fibrillation’ was the title of a famous paper by Wijffels et al. [11] in 1995, a hallmark in our understanding of AF. In a goat model of pacing-induced AF, these authors could demonstrate that with longer duration of stimulation, the induced AF lasted longer after switching off the stimulation until it became finally persistent and stimulation was no longer needed to maintain AF. Thus, the substrate for AF must have changed. According to our present knowledge, this remodeling process includes a reduction in the atrial effective refractory period (AERP) [11] due to ionic channel remodeling [18] with reduced  $I_{Ca,L}$  [18, 19], reduced sarcoplasmic  $Ca^{2+}$ -ATPase [20] as well as enhancement of some potassium channels (in human AF) (Kv4.3, Kv1.5, HERG, mink/KvLQT1, Kir3.1/Kir3.4, Kv6.2) which might be compensatory but insufficient [for a review, see ref. 21]. In contrast, in some cases an increase in  $I_{K1}$  and  $I_{K,ACh}$  has also been reported [22]. While the reduction in  $I_{Ca,L}$  seems to be common in AF in patients and in animal models of AF, the changes in potassium channels described above in humans are not found in this form in pacing-induced AF models [22, 23]. Moreover,  $I_{Na}$  has been reported to be reduced in pacing-induced AF in dogs [24].

Besides this electrical remodeling, structural remodeling occurs with myolysis, perinuclear glycogen storage, mitochondrial enlargement, loss of sarcoplasmic reticulum and loss of contractile proteins [for a review, see ref. 21]. The changes in contractile proteins lead to reduced contractility and may contribute to atrial dilatation and thus to increasing atrial stretch [25]. Moreover, at the tissue level, increasing fibrosis [26, 27] and deposition of amyloid have been described [12]. These changes will cause a reduction in AERP, mainly due to reduced  $I_{Ca,L}$ , and in atrial wavelength together with local conduction delay by enhanced nonuniform anisotropy [also see ref. 5] with heterogeneity structurally conserved by fibrosis, amyloid depositions and atrial dilatation, thereby favoring the occurrence of multiple wavelets and allowing re-entrant circuits of increasing complexity. Electrical remodelling occurs within several days, while structural and contractile remodeling takes several months.

These complex changes in cellular physiology and tissue structure are associated with changes in the intercellular networking by alterations of the expression and localization of intercellular gap junction channels.

## Role of Gap Junctions in Atrial Fibrillation

There is at present broad evidence that gap junction channels change in AF. In the atrium, mainly two isoforms are found, namely Cx43 and Cx40. In the present literature on gap junction alterations in AF, there are reports on changes in only one isoform, on reduced expression of one or more connexins as well as on increases in one or more connexins, depending on the patient population, accompanying diseases, or on the animal model used and depending on the atrial tissue investigated (right or left). However, despite this diversity, in nearly all reports, increased heterogeneity in the distribution of connexin has been observed with regional heterogeneity [28, 29] or with changes in the subcellular distribution and lateralization of connexins [30]. This increased regional heterogeneity and subcellular distribution changes (with lateralization of connexins) would fit into the enhanced nonuniformity and the augmented complexity of the wave patterns.

With regard to the initiation of AF, the role of gap junctions remains uncertain at present. However, a certain pattern of gap junction has been postulated to form the basis for the primary re-entrant circuits [9]. In >90% of the cases, electrophysiological catheter mapping in patients shows that the earliest activation at the beginning of an episode of AF starts at the ostium or within the pulmonary veins [10]. The first beats of the initiation of a paroxysm of AF have been assumed to be comparable to triggered activity triggering a re-entrant circuit [31]. However, at that point, the myocardial fiber arrangement of sleeves protruding 4–20 mm from the atrium into the veins comes into play. While in the study by Verheule et al. [9] there was no evidence from light or electron microscopy for a morphological difference between the myocytes in the sleeves and those in the atrium (i.e. these authors did not find the ‘pale cells’ postulated earlier by others as abnormal pulse generators [32]), Verheule et al. [9] found tight coupling between the venous and atrial myocardium with a circumferential organization of myocytes in the sleeves. In these structures Cx43 expression was comparable with atrial tissue while Cx40 was significantly lower. Particularly, the circumferential pattern may form a substrate for rapid circular re-entry. Similarly, Yeh et al. [33] also described a circumferential organization of myocytes but in the sleeves protruding from the atrium into the canine superior caval vein and postulated a role of this pattern and its heterogeneity (see below) in initiation of AF. However, they also observed atypical areas with abundant Cx43 expression surrounded by an area of Cx40 expression which was not seen in the pulmonary vein by Verheule et al. [9]. In addition, Yeh et al. [33] observed a colocalization of Cx43 with Cx40 or of Cx43 with Cx45 in other areas. In pulmonary veins, Cx45 has not been investigated so far. Nevertheless, while initiation of the first beat of an AF episode is still an open

question and may involve several contributing factors, a certain pattern of gap junction circumferential coupling in pulmonary and superior caval veins seems to provide the structural basis for a primary re-entrant circuit.

If the rest of the atrium exhibits features of increased heterogeneity in coupling with a certain current/sink ratio it can be assumed that such a primary circuit in the pulmonary vein may elicit waves spreading over the atrium and initiate AF. With increasing duration of AF the remodeling process will proceed and stabilize AF.

In this remodeling process, gap junctions also contribute to the chronification and stabilization of AF. Alterations of gap junctional coupling resulting in microscopic changes in the activation pattern have been postulated as a 'second factor' of tachycardia-induced atrial remodeling [34]. The findings in the literature are very heterogeneous with regard to connexin remodeling (see above). In the following, first the findings in humans will be outlined followed by observations in various animal models.

Thus, in humans suffering from AF, classified as lone AF, it has been reported by our group that in right atria Cx40 is upregulated while Cx43 is not or only slightly affected [30]. These patients were mostly patients undergoing cardiac bypass surgery. Polontchouk et al. [30] were the first to report that they found both connexins to be lateralized in AF, while in sinus rhythm these connexins are predominantly found at the cell poles (and almost no connexin is found at the lateral side). This lateralization of connexins has also been observed in humans (mostly in the right atrium) by others in subsequent studies with various changes in total connexin expression [35–37], so that lateralization of connexins might be a common feature in human AF. A later study of our group [38], on a total of 92 patients (lone AF:  $n = 41$ ; AF with mitral valve disease:  $n = 36$ ; sinus rhythm:  $n = 15$ ) revealed that in lone AF Cx40 was upregulated in the *left* atrium, while Cx43 was unchanged. In contrast, in patients suffering from AF associated with mitral valve disease both connexins, Cx40 and Cx43, were upregulated, indicating that underlying cardiac disease can modulate gap junctional remodeling, which must be taken into account. This study is especially interesting since it investigated the left atrium where AF often originates (see above). In the study by Yan et al. [35], the left atrium was investigated as well and upregulation of both Cx43 and Cx40 was found in AF, while in the right atrium only Cx40 was enhanced. A 2-fold increase in right atrial Cx40 has also been described in patients who developed postoperative AF indicating a pathophysiological role of the upregulation of this connexin [39]. Interestingly, Cx40 heterogeneity was seen in both AF and non-AF patients. On the other hand, Kostin et al. [36] found marked Cx40 heterogeneity in the right atrium in AF together with reduced Cx40 and Cx43 in the appendage, while in the free right atrial wall Cx40 was enhanced and Cx43 was reduced. As previously

seen by us, this was accompanied by lateralization of both connexins. Moreover, these authors found lateralization of N-cadherin, which has been discussed earlier to be involved in the docking of connexons.

In patients with rheumatic heart disease, Li et al. [37] described reduced Cx40, unaltered Cx43 levels, connexin lateralization and increasing inhomogeneity. Similarly, in patients with mitral valve disease, Nao et al. [40] found reduced Cx40, which was also inhomogeneous, but an increase in serine phosphorylation of Cx40 in the right atrium. Since mitral valve disease predominantly affects pressure, volume and stretch in the left atrium, the difference from the study of Wetzel et al. [38] (both connexins were upregulated in these patients in left atrium, see above) may be explained by the different tissue (right vs. left) and may point to a pathogenetic role of atrial pressure or stretch in modulating gap junctional remodeling. Regarding the genetic background, it is worth mentioning that about 16% of the male AF patients suffer from a familial form of AF, which has been mapped previously to chromosome 10q22-q23. However, a recent study could not detect any difference in the Cx40 gene sequence between these patients with familial AF and wild type Cx40 [41]. In contrast, in a more recent study polymorphisms of Cx40 were described and could be related to enhanced atrial vulnerability and increased risk of AF [42] (and see, the chapter by R. Hauer in this volume, p. 284).

In an interesting study, Kanagaratnam et al. [43] related right atrial mapping activation patterns to Cx40 expression in an excised area of the mapped region. In their AF patients (mostly with mitral valve disease or aortic valve disease), they found a reduction in *right* atrial Cx40 expression which correlated with the complexity of the wave pattern. However, although this is highly interesting and fits the hypothesis of increasing complexity of activation waves related to gap junctional remodeling, it cannot be excluded that this is an epiphenomenon of increased fibrosis in AF. Moreover, in the study by Kanagaratnam et al. [43], all patients of the control (i.e. sinus rhythm) group were patients with coronary heart disease undergoing coronary bypass grafting while most (12/13) of the AF patients were patients with either mitral valve disease (7/13) or aortic valve disease (5/13). Because of the possible influence of an underlying valvular disease on gap junctional remodeling, it is difficult to compare these two groups (however, as the author of this chapter knows from his own experience in humans, it is very difficult to find enough patients for such studies). Nevertheless, the study points to a possible pathophysiological relevance of connexin remodeling.

Taken together, these human studies show that there is considerable gap junctional remodeling, the level of expression of a certain connexin being possibly influenced by underlying diseases, patient selection and atrial tissue (right or left; appendage or free wall). Lateralization of connexins seems to be a

common phenomenon in human AF, and heterogeneity is described by a number of studies as well.

Since many detailed investigations are not possible in humans due to ethical and other reasons, animal models of atrial fibrillation are of highest interest (for an overview of models of AF, see: Schotten et al. [44]). A classical model is the famous goat model of Allesie's group in Maastricht [11], and its modifications. AF is induced by atrial tachycardic pacing in this model. In this model, left atrial activity is sensed and right atrial burst stimulation is delivered to maintain AF until it becomes persistent [11, 45]. A first study regarding connexin remodeling in this model [29] 'revealed no significant changes in the overall expression of Cx40 and Cx43 as a result of persistent AF'. However, the authors observed areas with low density of Cx40 surrounded by areas with normal Cx40 density, i.e. regional heterogeneity. In a later analysis, a reduction of the Cx40/Cx43 ratio (protein) has been described in both right and left atrial appendage [28]. Moreover, these authors found marked heterogeneity in immunohistologic Cx40 distribution. This reduction in Cx40/Cx43 was found early after induction of AF, but was not associated with a change in overall atrial conduction in atria defibrillated after AF, while conduction velocity was lower during sustained AF, so that the authors concluded that local inhomogeneities, microheterogeneities, increased number of sites with slower or blocked conduction may in part explain this difference [28]. In the goat model mentioned above, reverse remodeling was also described, after conversion of AF to sinus rhythm. Cx40 which was decreased in AF returned to normal within 2–4 months after conversion as the AERP normalized. Structural abnormalities such as myolysis or alterations of extracellular matrix were still present although less pronounced [45]. A similar decrease in atrial Cx40 has been observed in dogs subjected to occlusion of the left anterior descending coronary artery and subsequent (after 7 weeks) burst stimulation-induced AF [46].

In another study performed on dogs with right atrial tachycardic pacing-induced AF upregulation of right atrial Cx43 was observed [47]. In a subgroup of dogs, Elvan et al. [47] converted AF to sinus rhythm by radiofrequency catheter ablation and found that this enhanced Cx43 expression was absent in ablated areas or areas near the ablation zones. In accordance with other studies [30, 35–37], Elvan et al. [47] also observed an increase in side-to-side junctions in fibrillating atria.

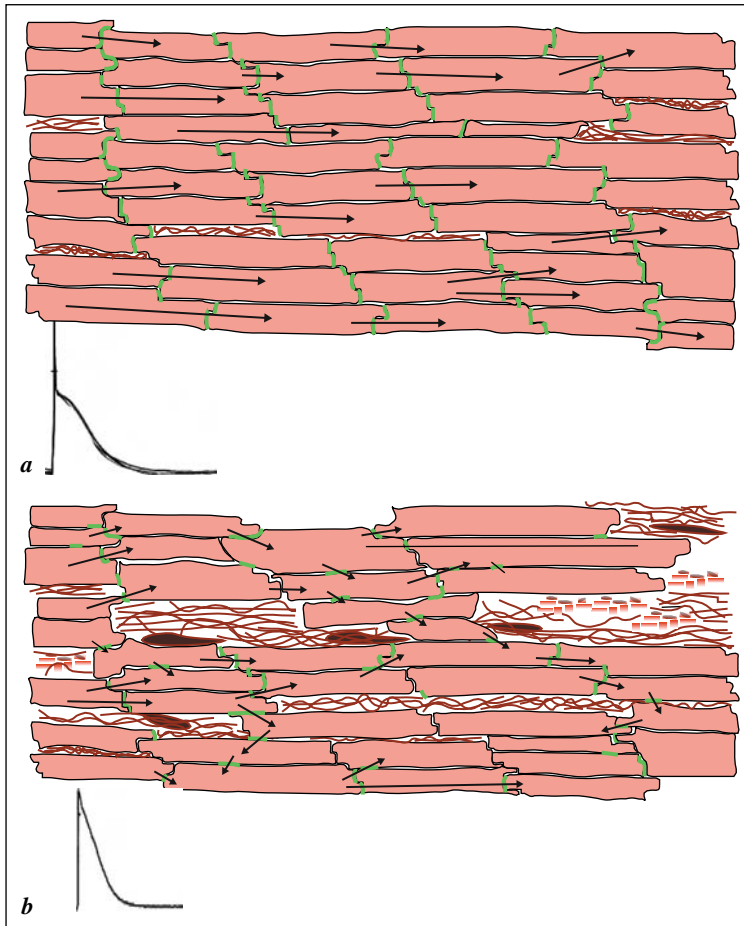
The fact that in the above animal models, the effects of AF on Cx43 and Cx40 expression are sometimes different from those in humans (which are heterogeneous in this regard as well), may be explained by species differences and – most importantly – by different pathomechanisms (electric pacing, valvular disease, myocardial infarction, etc.) and, in some models, different location of the primary source of AF (right vs. left atrium). Thus, the question which connexin



is up- or down-regulated may be influenced by underlying diseases, right vs. left atrium, electrical pacing, species, etc., while on the other hand nearly all authors agree that Cx expression becomes heterogeneous and many of them observe lateralization of connexins.

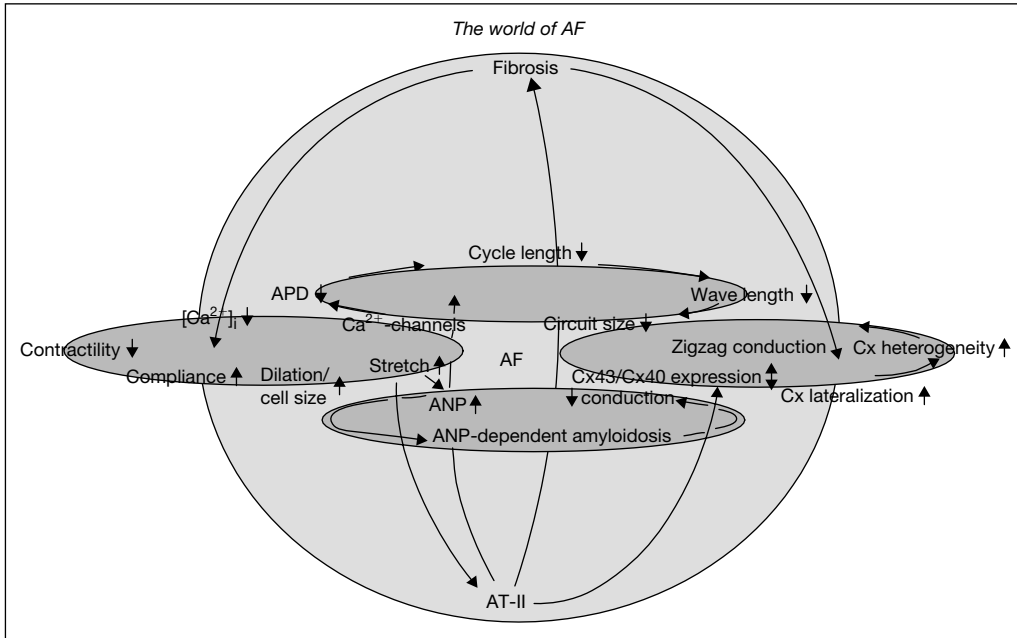
What is the biophysical outcome of these changes? This question was addressed by several studies. The lateralization of connexins observed by several authors (see above [30, 35–37] was also seen in rat atria submitted 10-Hz electrical stimulation for 24 h, which, in these specimens, led to an increase in transverse conduction velocity with a decrease in anisotropy [30]. In contrast, Koura et al. [5] found that age leads to an increase in connective tissue, resulting in enhanced transversal block and zigzag activation with extremely slow transverse conduction. On the other hand, atrial dilatation with enlarged myocytes has been observed in cats and dogs with mitral valve disease [48–50]. This also would change anisotropy. However, in an interesting computer model, Jongsma and Wilders [51], using a ventricular cell model, showed that a decrease in gap junction conductivity mainly affects transverse conduction and that longitudinal conduction is less sensitive. They concluded that cell size might be more important as was previously postulated by Spach et al. [52]. Assuming a shift of 40% of the gap junctions from polar membranes to lateral membranes would decrease longitudinal velocity from 65 to 58 cm/s and increase transverse velocity from 24 to 30 cm/s, thus decreasing anisotropy from 2.7 to 1.9 [51]. This 40% change in gap junctions is probably more than that observed experimentally by others (see above), although these also found a decrease in anisotropy due to increased transverse conduction. However, the computer model, although interesting, simplifies the reality, since it does not consider cellular enlargement, the increase in connective tissue, amyloid deposition, and especially assuming that all these connexins (being lateralized) form functional channels. The situation is probably more complex and can be characterized by the coexistence of lateralization of connexins, changes in the level of connexin isoform expression, regional heterogeneity, enlargement of myocytes, lateral but inhomogeneous separation of myocytes by collagen or amyloid (fig. 1). The regional heterogeneity of these changes is probably one of the critical factors forming the biophysical basis for AF.

Which pathophysiological mechanisms may contribute to the gap junctional remodeling process in AF? There are a number of potentially relevant stimuli including local ischemia, stretch, angiotensin, calcium, electrical activity and altered extracellular matrix. Among these, it has been demonstrated that ventricular ischemia leads to decreased expression of Cx43 [53]. AF can triple oxygen consumption [54]. If AF leads to local ischemia due to enhanced cellular activity or to a concomitant ischemic heart disease, a similar downregulation of Cx43 may occur in ischemic regions of the atria. Ischemia has been shown to



**Fig. 1.** Scheme of the changes in tissue structure and gap junctional communication involved in atrial fibrillation. For details, see text.

promote AF in a dog model [55]. Occlusion of an atrial coronary artery led to a prolongation of the duration of burst-stimulation-induced AF. In support of this hypothesis, Thijssen et al. [56] reported in the goat AF model on an up-regulation of the hypoxia-inducible factor HIF1 $\alpha$ , which is a typical response to ischemic stress, and may induce expression of a large number of genes. In contrast, others did not detect any signs of atrial ischemia despite alteration in Cx expression with decreased Cx40 [46]. Another factor which might contribute to the remodeling process is intracellular calcium (initially  $[Ca^{2+}]_i$  is increased in short episodes of AF) and reduction in  $I_{Ca,L}$  which has been supposed to be



**Fig. 2.** Scheme of the pathophysiological mechanisms involved in the remodeling process in atrial fibrillation and their participation in gap junctional remodeling. AT-II = Angiotensin II. For details, see text.

involved in atrial stunning and dilatation in AF [for reviews, see ref. 21, 25]. Regarding connexins, we could show that a calcium antagonist can reduce Cx43 and Cx40 expression in cultured rat cardiomyocytes [57]. At present, it is not known, whether the intracellular calcium concentration can regulate atrial connexin expression in AF. However, it might be a contributing factor. Similarly, stretch may influence the cells differentiation as is known from a number of studies in cultured cells. Thus, it might be assumed that enhanced atrial stretch might contribute to the remodeling process although it is unknown at present whether this parameter can influence atrial connexin expression. The alteration of the extracellular matrix reported in humans [26] may also influence protein synthesis or differentiation of the cells. Another interesting factor which might be involved in connexin remodeling is angiotensin which has been reported to be enhanced in canine AF in congestive heart failure as well as ERK1/2 [58]. In addition, angiotensin can enhance Cx43 expression in cardiomyocytes via stimulation of AT<sub>1</sub> receptors, which can be inhibited by losartan [59] activating ERK1/2 and p38MAPK. Interestingly, we also could show that in human *left* atrium (*but not right*) AT<sub>1</sub> receptors are up-regulated in AF [60], while in right atrium Goette et al. [61] found a down-regulation of AT<sub>1</sub> receptors. Since the left atrium often

resembles the source of AF this up-regulation of AT<sub>1</sub> receptors might be of pathophysiological relevance. Moreover, since enhanced AT<sub>1</sub> and increased AT-II would augment the AT<sub>1</sub>-dependent signal transduction, and since AT<sub>1</sub> regulates Cx43, it can be imagined that enhanced AT<sub>1</sub> stimulation may account for an up-regulation of Cx43 in certain types of AF. Interestingly, others could show that angiotensin-converting enzyme inhibition by enalapril was effective to prevent from AF-induced increases in fibrosis, conduction heterogeneity, AT-II expression and ERK expression [58]. Moreover, Sakabe and co-workers could demonstrate that AF-induced lateralization of Cx43 (see above) could also be prevented with enalapril suggesting a role for angiotensin II in connexin remodeling [27]. Application of ACE-inhibitors or AT<sub>1</sub> antagonists might provide new therapeutic approaches for the treatment of AF (especially after cardioversion). Figure 2 gives a schematic overview on the factors discussed in atrial gap junction remodeling.

## Concluding Remarks

Thus, gap junctions are involved in AF at different levels. A common aspect is the geometric distribution of gap junctions within the atrial tissue and within a cell providing a certain biophysical network. Since angiotensin II seems to be involved in this process ACE inhibitors or AT<sub>1</sub> antagonists may interfere with gap junctional remodeling and help to maintain sinus rhythm.

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## Connexins in the Sinoatrial and Atrioventricular Nodes

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### Abstract

The sinoatrial node (SAN) and the atrioventricular node (AVN) are specialized tissues in the heart: the SAN is specialized for pacemaking (it is the pacemaker of the heart), whereas the AVN is specialized for slow conduction of the action potential (to introduce a delay between atrial and ventricular activation during the cardiac cycle). These functions have special requirements regarding electrical coupling and, therefore, expression of connexin isoforms. Electrical coupling in the center of the SAN should be weak to protect it from the inhibitory electrotonic influence of the more hyperpolarized non-pacemaking atrial muscle surrounding the SAN. However, for the SAN to be able to drive the atrial muscle, electrical coupling should be strong in the periphery of the SAN. Consistent with this, in the center of the SAN there is no expression of Cx43 (the principal connexin of the working myocardium) and little expression of Cx40, but there is expression of Cx45 and Cx30.2, whereas in the periphery of the SAN Cx43 as well Cx45 is expressed. In the AVN, there is a similar pattern of expression of connexins as in the center of the SAN and this is likely to be in large part responsible for the slow conduction of the action potential.

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The sinoatrial node (SAN) is the pacemaker of the heart [1]. The action potential spontaneously generated in the SAN propagates through the atria to reach the atrioventricular node (AVN). The AVN is the only normal conduction pathway between the atria and ventricles and it delays the conduction of the



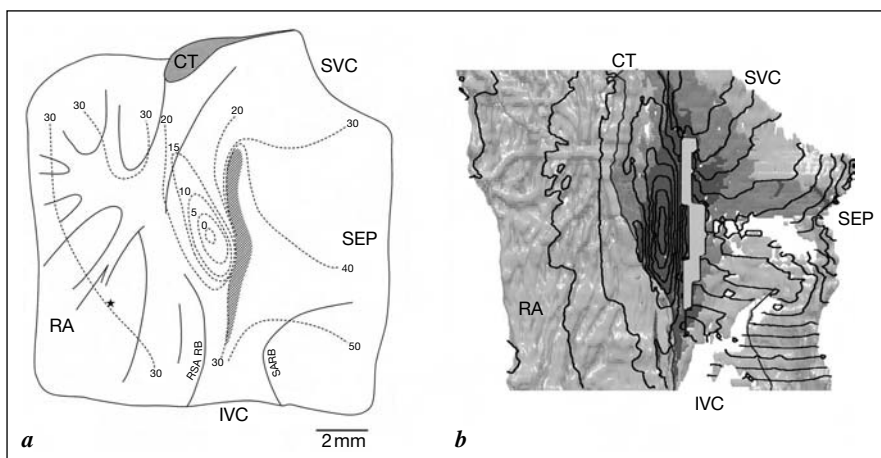
action potential between the atria and ventricles [2]. This delay is important, because it ensures that atrial systole is complete before ventricular systole begins. Once the action potential has propagated through the AVN, it passes rapidly through the His-Purkinje system to reach the ventricles, causing a coordinated contraction of the two chambers. In order to fulfill their specialized functions, the SAN and AVN have (i) specialized structures, (ii) a different pattern of expression of connexins and, as a result, different electrical coupling between cells and (iii) a different pattern of expression of ion channels and, consequently, different action potential configurations (as compared to the working myocardium). This chapter is concerned with the specialized pattern of expression of connexins in the SAN and AVN and how this impacts on function.

### **Sinoatrial Node**

Figure 1a shows the activation sequence of the rabbit SAN. In the rabbit, the SAN lies in the intercaval region between the superior and inferior vena cava. It is bounded on one side by the atrial muscle of the crista terminalis (a thick muscle bundle) and on the other side by the atrial muscle of the interatrial septum. As shown by the 0-ms isochrone in figure 1a, the leading pacemaker site lies in the center of the SAN,  $\sim 0.5\text{--}2\text{ mm}$  from the crista terminalis in the intercaval region [1]. The action potential preferentially propagates from the leading pacemaker site in an oblique cranial direction (i.e. conduction of the action potential is faster in this direction). The action potential first propagates to the periphery of the SAN, where the SAN meets the atrial muscle (the border between two tissues occurs on the endocardial surface of the crista terminalis). The action potential then propagates into the atrial muscle of the crista terminalis and right atrial free wall. Figure 1a shows that from the center to the periphery of the SAN conduction is slow (i.e. the isochrones are closely spaced), but in the atrial muscle of the crista terminalis and right atrial free wall conduction is faster (i.e. the isochrones are widely spaced). Towards the interatrial septum, conduction of the action potential is blocked and the shaded area in figure 1a is the block zone. For the atrial potential to propagate to the interatrial septum, it must propagate around the block zone. The activation sequence of the SAN in other species is similar, although there are species differences [1].

#### *Theoretical Requirements of the Sinoatrial Node for Electrical Coupling*

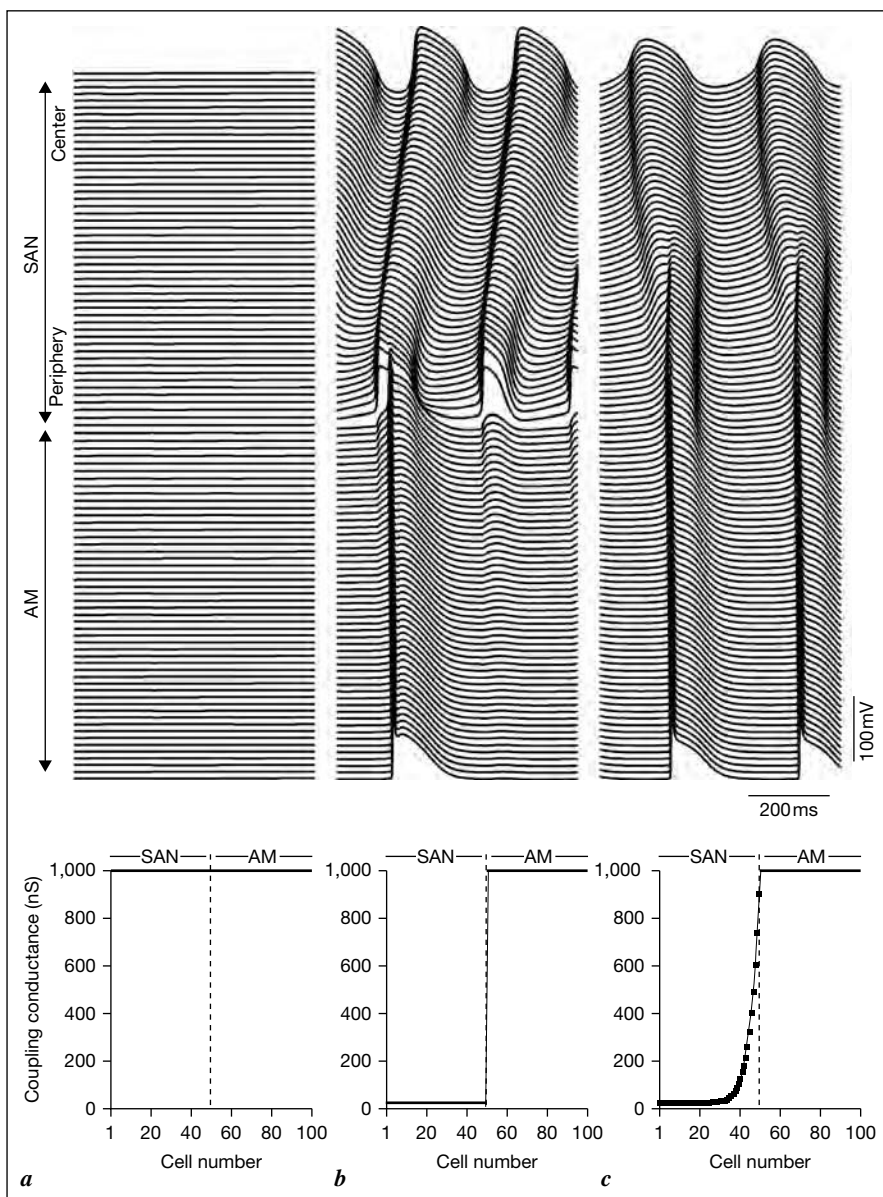
In order for the SAN to drive the surrounding atrial muscle, it must be electrically coupled to it. However, the atrial muscle is more hyperpolarized than the SAN and it does not show pacemaking (unlike the SAN) and because



**Fig. 1.** Activation sequence of the rabbit SAN. **a** Experiment. The dashed lines are isochrones at the time (in ms) shown and the shaded area is the conduction block zone. **b** Simulation [Inada, unpubl. data]. The solid lines are isochrones at equal (but arbitrary) time intervals and the shaded area (in the equivalent position as the shaded area in **a**) is the conduction block zone. Light grey = Atrial muscle; mid-grey = peripheral SAN tissue; dark grey = central SAN tissue; CT = crista terminalis; IVC = inferior vena cava; LSARB = left sinoatrial ring bundle; RA = right atrial free wall; RSARB = right sinoatrial ring bundle; SEP = interatrial septum; SVC = superior vena cava.

of the electrotonic interaction between the two tissues (as a result of the electrical coupling) the pacemaker activity of the SAN tends to be suppressed. There are two lines of evidence for this: first, when the atrial muscle is cut from the SAN, there is an acceleration of the pacemaker activity of the SAN [3–5]. Secondly, if an atrial cell is coupled to an SAN cell, the pacemaker activity of the SAN cell is slowed or abolished [5–9].

It is probable that the SAN is organized in such a way that the suppressive effect of the atrial muscle is minimized and yet the SAN is still able to drive the atrial muscle. Joyner and van Cappelle [10] addressed this issue using mathematical modeling. They suggested that ‘the SAN should be a minimum size’, because the SAN must have sufficient capacity to withstand the hyperpolarizing influence of the atrial muscle and to provide sufficient depolarizing current to drive the atrial muscle. The space constant ( $\lambda$ ) is the distance over which an electrotonic potential, caused by intracellular current injection, decays to  $1/e$  ( $\sim 37\%$ ) of its original value. In the rabbit, the space constant of the SAN, perpendicular to the crista terminalis, is  $0.2\text{--}0.3\ \mu\text{m}$  [1], whereas the leading pacemaker site is  $\sim 0.5\text{--}2\text{ mm}$  from the crista terminalis. Therefore, the leading



**Fig. 2.** Requirements of the SAN for electrical coupling. Results from a one-dimensional model of the rabbit SAN and surrounding atrial muscle (AM) are shown [Inada and Honjo, unpubl. data]. The model consists of a string of 50 SAN cells (extending from the center to the periphery of the SAN) connected to a string of 50 atrial cells. The SAN action potentials were simulated using the model of Zhang et al. [51]. In their model, ionic current

pacemaker site is located  $\sim 2$  or more space constants from the atrial muscle and the suppressive effect of the atrial muscle on the SAN should be minimal.

Joyner and van Cappelle [10] also reported that ‘electrical coupling should be weak within the SAN’, in order to minimize the electrotonic interaction between the atrial muscle and SAN. Finally, they suggested that ‘there should be an increase in electrical coupling in the periphery of the SAN’, in order that the SAN can provide sufficient depolarizing current to drive the atrial muscle. Figure 2 shows results from a one-dimensional model of the SAN and surrounding atrial muscle [Inada and Honjo, unpubl. data]. In the first case (fig. 2a), the coupling conductance was high both in the SAN and atrial muscle (see graph in fig. 2a) – as a result of the suppressive effect of the atrial muscle, the SAN was quiescent. In the second case (fig. 2b), the coupling conductance was low throughout the SAN, but high in the atrial muscle – the low coupling conductance in the SAN protected the SAN from the suppressive influence of the atrial muscle and, therefore, pacemaker activity was observed in the SAN. However, the SAN was unable to drive the surrounding atrial muscle in a robust manner: some action potentials failed to exit from the SAN and some action potentials did propagate into the atrial muscle from the SAN, but only after a delay (fig. 2b). Furthermore, the action potential was first initiated in the periphery of the SAN (the intrinsic pacemaker activity of the periphery is faster than that of the center) and from here it propagated into the center of the SAN, whereas it is known that the center is the leading pacemaker site under normal physiological conditions. In the final case (fig. 2c), the coupling conductance rose from a low value in the centre of the SAN to a high value in the periphery of the SAN (once again the coupling conductance was high in the atrial muscle) – in this case the SAN showed pacemaking and it stimulated the atrial muscle. The action potential was first initiated in the centre of the SAN (electrotonic suppression by the atrial muscle suppressed pacemaking in the periphery) and from here it propagated into the periphery of the SAN and then (without an abnormal delay) into the atrial muscle. This corresponds to the normal physiological case. Experimental evidence of weak electrical coupling in the center of the SAN and stronger electrical coupling in the periphery is considered in the next sections.

densities are functions of the cell capacitance and in the simulations shown cell capacitance was assumed to rise from the center to the periphery. The atrial action potential was simulated using one of the suite of Oxsoft HEART models. The membrane potential of all 100 cells in the one-dimensional model is shown in three cases: when the coupling conductance between neighboring cells was high throughout the SAN and atrial muscle (**a**), when the coupling conductance was low in the SAN, but high in the atrial muscle (**b**), and when the coupling conductance rose from the center to the periphery of the SAN (high coupling conductance in the atrial muscle (**c**). The graphs show the coupling conductance for all 100 cells in the one-dimensional model, the 50 SAN cells and the 50 atrial cells.

### *Weak Electrical Coupling in the Center of the Sinoatrial Node*

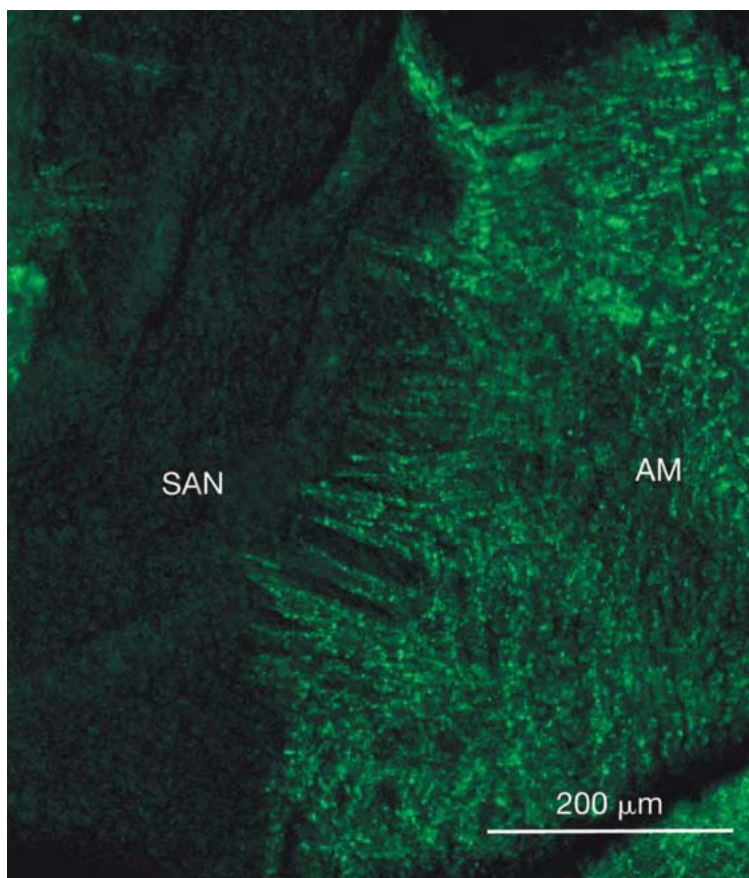
Three lines of evidence show that electrical coupling in the SAN is weak as suggested by Joyner and van Cappelle [10]. The conduction velocity of the action potential is dependent on electrical coupling and the conduction velocity is low in the center of the SAN: in the rabbit, the conduction velocity in the center of the SAN is 4.5 cm/s parallel to and 3.0 cm/s perpendicular to the crista terminalis, whereas in the periphery of the SAN the conduction velocity is higher (49.7 cm/s parallel to and 36.3 cm/s perpendicular to the crista terminalis) [1]. The space constant is another measure of electrical coupling and the space constant of the SAN is lower than that of the atrial muscle [1]. Finally, for the rabbit, the gap junction conductance between pairs of SAN cells, atrial cells and ventricular cells has been measured to be  $\sim 3$ –8,  $\sim 169$  and  $\sim 175$  nS, respectively [11, 12].

### *Gap Junctions in the Center of the Sinoatrial Node*

Electron microscopy shows the presence of gap junctions in the SAN. Masson-Pévet et al. [13] reported that the gap junctions of the rabbit SAN are smaller and about ten times less numerous than in working myocardium. Saffitz et al. [14] also reported that the gap junctions of the dog SAN are smaller and less numerous than in the working myocardium: in the SAN, atrial muscle of the crista terminalis and left ventricle, the mean length of gap junction profiles was  $\sim 0.17$ ,  $\sim 0.28$  and  $\sim 0.82$   $\mu\text{m}$ , respectively; the mean length of intercalated disk (per 100  $\mu\text{m}^2$  cell area) was 2.6, 4.4 and 3.5  $\mu\text{m}$ , respectively, and the mean number of gap junction profiles (per 100  $\mu\text{m}$  of intercalated disk) was 5.6, 11.5 and 15.5, respectively. The smallness and low number of gap junctions in the SAN is consistent with the weak electrical coupling in the SAN.

### *Expression of Connexins 30.2, 40, 43 and 45 in the Center of the Sinoatrial Node*

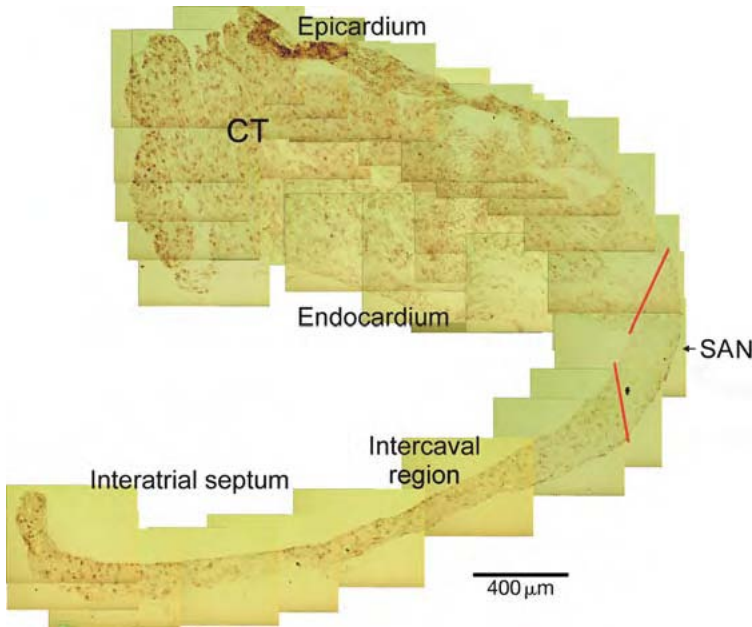
Gap junctions consist of clusters of channels linking two neighboring cells. Each channel consists of a pair of linked hemichannels (one in each of the opposing cell membranes). Each hemichannel (connexon) is composed of six connexins. Connexins are a multigene family and Cx30.2, Cx37, Cx40, Cx43, Cx45 and Cx46 are present in the heart. Using immunohistochemistry, the expression of connexin proteins has been studied in the SAN. Cx43 forms medium conductance (60–100 pS [15]) gap junction channels and is abundant in the working myocardium. In a wide range of species (mice, rats, guinea pigs, rabbits, dogs, cows and humans), Cx43 has been reported to be absent in the SAN [11, 16–23], although there are two conflicting reports [12, 24]. An example is shown in figure 3 [Liu and Lei, unpubl. data]. Figure 3 shows immunolabeling of Cx43 in the mouse SAN at the border of the SAN with the atrial



**Fig. 3.** Interdigitating fingers of Cx43-negative and Cx43-positive cells at the border of the SAN and atrial muscle (AM) in the mouse. A whole SAN-atrial muscle preparation from the mouse was immunolabeled for Cx43 and the image shown shows Cx43 labeling (green signal) at the border of the SAN with the atrial muscle towards the interatrial septum [Liu and Lei, unpubl. data].

muscle towards the interatrial septum. The absence of Cx43 protein in the SAN is the result of a lack of Cx43 mRNA: figure 4 shows labeling of Cx43 mRNA by in situ hybridization in a section through the SAN of the rat [Dobrzynski, unpubl. data]. In the atrial muscle, on either side of the SAN, there is labeling (in the form of rings around the nucleus, corresponding to the rough endoplasmic reticulum where the mRNA is being processed), but in the SAN (between the red lines) there is none.

Cx45 forms small conductance (20–40 pS [15]) gap junction channels. Based on immunolabeling, Coppen et al. [22] reported the presence of Cx45 in the center



**Fig. 4.** Absence of Cx43 mRNA in the rat SAN. A section cut perpendicular to the crista terminalis through the crista terminalis and intercaval region (where the SAN is located) is shown [Dobrzynski, unpubl. data]. The section was labeled for Cx43 mRNA using in situ hybridization. The labeling is in the form of rings (the rings appear as spots at low magnification) around the nuclei. There is labeling in the atrial muscle, but not in the SAN (between the red lines). CT = Crista terminalis.

of the SAN in the rabbit. The Cx45 spots in the SAN were small and sparse as compared to the Cx43 spots in the neighboring atrial muscle. This is consistent with the size and density of gap junctions in the SAN as observed in electron microscopy. Honjo et al. [25] obtained comparable data using isolated rabbit SAN cells. Davis et al. [18, 19] also reported the presence of Cx45 in the dog and human SAN. The absence of the medium conductance gap junction channel protein, Cx43, and the presence of the small conductance gap junction channel protein, Cx45, is consistent with weak electrical coupling in the SAN.

Cx40 forms large conductance (200 pS [15]) gap junction channels and Cx40 has been reported to be present in the SAN of rabbits, dogs and humans [11, 18, 19, 22, 25]. The presence of Cx40 appears to be inconsistent with weak electrical coupling in the SAN. However, there are reasons why this may not be so. Coppen et al. [22] noted that the Cx40 spots in the center of the SAN in the rabbit were small and sparse as compared to the Cx43 spots in the neighboring atrial muscle, whereas Verheule et al. [11] noted that the Cx40 spots in the center

of the rabbit SAN were small and sparse as compared to the Cx40 spots in the neighboring atrial muscle. Furthermore, using quantitative PCR we have shown that the abundance of mRNA for Cx40 in the rabbit SAN is ~54% of that in the neighboring atrial muscle [Tellez and Dobrzynski, unpubl. data]. Finally, the SAN is rich in fibroblasts, and Camelliti et al. [26] reported that Cx40 couples fibroblasts rather than myocytes (whereas Cx45 couples myocytes). Intriguingly, Camelliti et al. [26] also reported that Cx45 couples fibroblasts to myocytes.

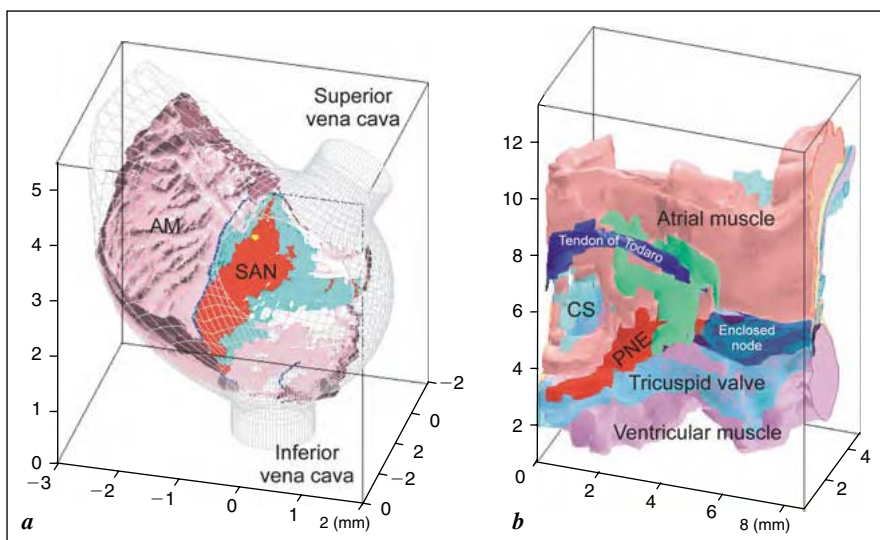
Cx30.2 forms small conductance (~9 pS) gap junction channels and is mainly expressed in the cardiac conduction system including the SAN [27]. The presence of homotypic gap junction channels composed of Cx30.2 is of course consistent with the weak electrical coupling in the SAN. However, intriguingly, Cx30.2 forms functional heterotypic gap junction channels with Cx40, Cx43 and Cx45 and the heterotypic gap junction channels all exhibit small conductances (15–18 pS) [27]. Therefore, even if Cx40 is expressed in the SAN in significant amounts it may not form large conductance gap junction channels. Finally, the heterotypic gap junction channels show marked rectification and this could result in a directional asymmetry in electrical coupling. For example, such rectifying heterotypic channels could allow minimal suppression of the SAN by the atrial muscle, but still allow the SAN to drive the atrial muscle. Such ideas need to be explored experimentally.

Verheule et al. [11] reported the presence of Cx46 in the center of the rabbit SAN. The functional significance of this has yet to be investigated.

#### *Expression of Connexins in the Periphery of the Sinoatrial Node*

As discussed above, according to Joyner and van Cappelle [10], if the SAN is to drive the surrounding atrial muscle, there should be a gradual increase in electrical coupling in the periphery of the SAN. At the border of the SAN and atrial muscle, ten Velde et al. [20] were the first to show (in the guinea pig) the presence of an intermingling or interdigitation of strands of Cx43-positive atrial cells and Cx43-negative SAN cells. A similar intermingling has been reported in rats, rabbits, dogs, cows and humans [17, 21, 26, 28] as well as mice [Liu and Lei, unpubl. data] (fig. 3). Coppen et al. [22] reported that, in the rabbit, both Cx43 and Cx45 are expressed in the periphery of the SAN. Recently, Dobrzynski et al. [28] refined these observations: they used neurofilament to distinguish between atrial cells and SAN cells (in the rabbit, neurofilament is exclusively expressed in the cardiac conduction system [21]). Dobrzynski et al. [28] showed that, in the periphery of the SAN in the rabbit, there are Cx43-negative/Cx45-positive/neurofilament-positive SAN cells (as in the center of the SAN), but there are also Cx43-positive/Cx45-negative/neurofilament-negative atrial cells and Cx43-positive/Cx45-positive/neurofilament-positive SAN cells [11]. In support of these observations, Honjo et al. [25] (see also Verheule et al. [11])





**Fig. 5.** Anatomical models of the SAN and AVN of the rabbit. **a** Model of the SAN [28]. The model of the SAN and surrounding atrial muscle (AM) is embedded in an idealized wire-frame model of the right atrium. Pink = Atrial muscle; blue = peripheral SAN tissue; red = central SAN tissue. **b** Model of the AVN [49]. A tract of nodal tissue is located at the atrio-ventricular junction. The enclosed part of the AVN is shown in purple and this is continuous with the posterior nodal extension (PNE) shown in red. The posterior nodal extension may form the slow pathway into the AVN. Loosely packed atrial muscle is shown in green and this may form (or be part of) the fast pathway into the AVN. CS = Coronary sinus.

showed that large SAN cells (likely to be from the periphery of the SAN) express both Cx43 and Cx45 (whereas small SAN cells, likely to be from the center of the SAN, express only Cx45). An analogous transitional structure with both Cx43 and Cx45 has been reported in the mouse [23].

In summary, the zone of increased electrical coupling proposed by Joyner and van Cappelle [10] could be the result of (i) the intermingling of Cx43-positive atrial cells and Cx43-negative SAN cells at the border of the two tissues and (ii) the expression of both Cx43 and Cx45 in SAN cells in the periphery of the SAN.

#### *Model of the Sinoatrial Node*

Dobrzynski et al. [28] have recently constructed a model (a mathematical array) of the rabbit SAN and some of the surrounding atrial muscle (shown in fig. 5a). A rabbit SAN-atrial muscle preparation like that shown in figure 1a was sectioned perpendicular to the crista terminalis from the superior to the inferior vena cava, and every  $\sim 200 \mu\text{m}$ , sections were stained with Masson's trichrome

(to show the histology) and immunolabeled for Cx43 and neurofilament. These stained sections were used to reconstruct the geometry of the preparation and the distribution of myocytes, and the immunolabeling was used to distinguish between atrial and SAN cells. In figure 5a, the pink zone corresponds to atrial muscle (it exclusively contains Cx43-positive/neurofilament-negative cells), the blue zone corresponds to the periphery of the SAN (it contains a mixture of cell types: Cx43-positive/neurofilament-negative, Cx43-negative/neurofilament-positive and Cx43-positive/neurofilament-positive cells – see above for more details) and the red zone corresponds to the center of the SAN (it exclusively contains Cx43-negative/neurofilament-positive cells). As discussed above, the periphery of the SAN may be the zone of increased electrical coupling proposed by Joyner and van Cappelle [10] and figure 5a shows that the blue zone with its mix of cell types lies between the center of the SAN (red) and the atrial muscle (pink) around the whole of the perimeter of the SAN.

If we understand the geometry of the SAN and the factors determining the conduction of the action potential, then we should be able to reconstruct the activation sequence of the SAN. Figure 1b shows such a simulation [Inada, unpubl. data]. The geometric model of the rabbit SAN of Dobrzynski et al. [28] was used. Conduction of the action potential was calculated using a cellular automaton model. With this simple model, the tissue was divided into  $\sim 2.5$  million ‘nodes’ (groups of cells). A node was assumed to become active (in the next time step) if any node within a critical radius,  $R$ , of the node of interest was active. Coupling was assumed to be weaker in the SAN than in the atrial muscle ( $R$  was fourfold less in the periphery of the SAN and eightfold less in the center of the SAN than in the atrial muscle). As a result, in the simulation (fig. 1b), conduction of the action potential in the SAN was slow (i.e. the isochrones are close together), whereas conduction in the atrial muscle was fast (i.e. the isochrones are widely spaced), as observed experimentally (fig. 1a).

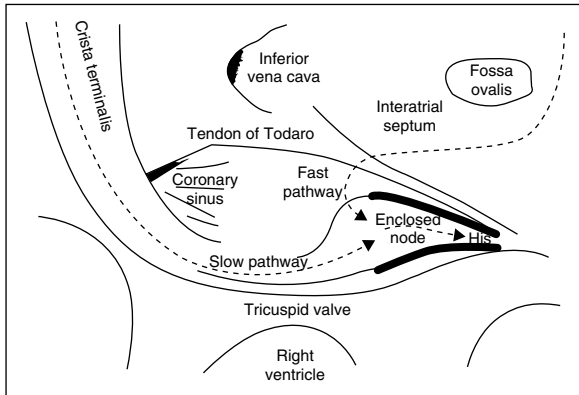
Cell orientation is also a major determinant of the conduction velocity (cardiac muscle is anisotropic – ‘anisotropy’ refers to changes in conduction velocity dependent on the orientation of the cells). Cardiac muscle is anisotropic, because the action potential jumps almost instantaneously from one end of a cell to another and the rate-limiting step for conduction of the action potential through a tissue is the transmission of the action potential across the gap junctions connecting cells. Because these ‘jumps’ are greater along the length of a cell (than across the width), the conduction velocity will tend to be greater in a direction parallel to the long axis of a cell than perpendicular to it. However, the anisotropy will also depend on the ratio of the end-to-end versus side-to-side gap junctional coupling conductances. For example, Saffitz et al. [29] showed in the dog that in the left ventricular myocardium the ratio of longitudinal to transverse conduction velocities is  $\sim 3$ , whereas it is  $\sim 10$  in the crista terminalis in the right atrium. There are

approximately equal numbers of end-to-end and side-to-side connections between ventricular cells, whereas  $\sim 80\%$  of connections between atrial cells of the crista terminalis are end-to-end connections [29]. In summary, to calculate the activation sequence of the SAN, both cell orientation and the ‘anisotropy ratio’ (incorporating both the effect of cell orientation per se and the ratio of end-to-end and side-to-side coupling conductances) must be taken into account. The simulation shown in figure 1b includes cell orientation [from 28]: in the crista terminalis and the pectinate muscles in the right atrial free wall, cells were assumed to run parallel to the muscle bundles; in the SAN, cells were assumed to run parallel to the crista terminalis, and towards the interatrial septum, cells were assumed to run perpendicular to the crista terminalis. The simulation shown in figure 1b also includes an anisotropy ratio of 10 (i.e.  $R$  was 10-fold greater in the direction longitudinal to a cell than transverse to it). As a result, in the simulation (fig. 1b), conduction of the action potential in the SAN occurred faster parallel to the crista terminalis as observed experimentally (fig. 1a).

Finally, the simulation in figure 1b includes a conduction block zone (the shaded area in fig. 1b). In the simulation, the nodes were assumed to be poorly excitable in the block zone [1]. As a result of the block zone, in the simulation (fig. 1b), conduction of the action potential occurred around the top and bottom of the block zone to reach the interatrial septum as observed experimentally (fig. 1a).

#### *Aging and Expression of Connexins in the Sinoatrial Node*

During aging in the human and other mammals, the functioning of the SAN declines and there is a decrease in the intrinsic heart rate (heart rate in the absence of autonomic nerve stimulation) and an increase in the SAN conduction time (the time for the action potential to propagate out of the SAN) [30]. Sick sinus syndrome is a dysfunction of the SAN, and symptoms include severe sinus bradycardia, sinus pauses or arrest, sinoatrial exit block, chronic atrial tachyarrhythmias, and alternating periods of atrial bradyarrhythmias and tachyarrhythmias, and inappropriate heart rate responses during exercise or stress [31]. The incidence of sick sinus syndrome increases markedly with age [31] and sick sinus syndrome could be related to the decline in function in the SAN with age. In the guinea pig, Jones et al. [32] reported that the size of the Cx43-negative region of the SAN increases in size with age from 0 to 38 months of age (birth to senescence). For example, it increased from  $\sim 4$  to  $\sim 48 \text{ mm}^2$  ( $\sim 14$ -fold increase) from 1 to 38 months of age. Part of this could simply be the result of an increase in the size of the heart with age. However, from the age of 18 months, body and heart size were approximately constant and yet there was still a substantial  $\sim 2$ -fold increase in the size of the Cx43-negative region from  $\sim 22$  to  $\sim 48 \text{ mm}^2$  [32]. The reason for the increase is not known (it could be the



**Fig. 6.** Pathways into the AVN. A schematic diagram of the rabbit AVN is shown with major landmarks and the fast and slow pathways labeled. Adapted from Mazgalev and Tchou [33] and Billette [52].

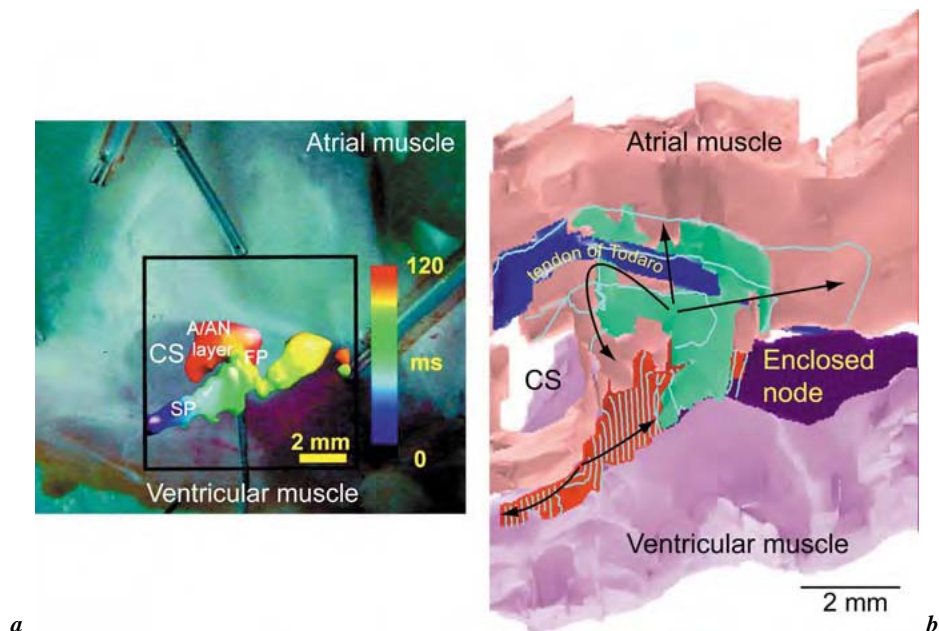
result of a proliferation of Cx43-negative myocytes, a spreading of Cx43-negative myocytes (but no increase in their number) or a loss of Cx43 expression by former Cx43-positive myocytes). In the older animals, the SAN conduction time was greater as expected (it increased  $\sim 2$ -fold from  $\sim 9$  to  $\sim 21$  ms from 0 to 38 months of age) and was primarily the result of the greater distance of Cx43-negative tissue the action potential had to propagate across to reach the atrial muscle from the leading pacemaker site in the center of the SAN (this distance increased 2-fold from  $\sim 1.3$  to  $\sim 2.6$  mm from 0 to 38 months of age) [32].

### Atrioventricular Node

The AVN lies within the triangle of Koch bounded by the coronary sinus, tendon of Todaro and tricuspid valve (fig. 6). The enclosed part of the AVN (it is enclosed within the connective tissue of the central fibrous body), including the compact part of the AVN, lies at the apex of the triangle (fig. 6). The action potential enters the enclosed part of the AVN via two principal pathways: a slow pathway located more posteriorly and inferiorly at the base of the triangle of Koch close to the root of the tricuspid valve and a fast pathway located more anteriorly and superiorly (fig. 6). It has been argued that the fast pathway is the normal conduction pathway into the enclosed node during sinus rhythm, although this is not certain [33]. The principal function of the AVN is to delay the conduction of the action potential from the atria to the ventricles. In the

rabbit, the action potential propagates through the atrial muscle (and transitional atrionodal, AN, cells) in the triangle of Koch region at a velocity of  $\sim 35.1$  cm/s, whereas along the slow pathway and enclosed node the conduction velocity of the action potential is less ( $\sim 7.4$  cm/s) as the name of the pathway implies [34]. The conduction velocity of the fast pathway is not known [33] and the name of the pathway comes from the shorter conduction time of the action potential via this pathway (the short conduction time could be the result of a low conduction velocity or a short pathway). The delayed conduction in the AVN must be the result of the slowness of conduction through the fast and slow pathways and/or the slowness of conduction through the enclosed node.

The so-called dual pathway electrophysiology of the AVN is important: it is the presence of the two pathways into the enclosed part of the AVN that is thought to give rise to AVN reentrant arrhythmias, because they present a circular pathway around which the action potential is able to propagate. AVN reentrant tachycardia (AVNRT) is considered to be the most common form of paroxysmal supraventricular tachycardia (except atrial fibrillation) in adults [35]. AVN reentrant arrhythmias can be of three types: typical AVNRT is slow/fast and involves anterograde conduction along the slow pathway, retrograde conduction along the fast pathway and conduction through the atrial muscle back to the slow pathway [34]. Atypical AVNRT is either fast-slow (involving anterograde conduction along the fast pathway, retrograde conduction along the slow pathway and conduction through the atrial muscle back to the fast pathway) or slow-slow (involving intranodal pathways) [34, 35]. In the case of slow/fast and fast/slow reentry, the circular pathway is able to sustain reentry, because propagation of the action potential along the slow pathway (at least) is slow: the conduction time along the slow pathway (70–130 ms in the rabbit [34]) is equal to the effective refractory period of the tissue ( $\sim 100$  ms in the rabbit [36]). Therefore, by the time the action potential has completed a reentry circuit, the tissue will have recovered from refractoriness and the reentry will be sustained. To initiate reentry there must be unidirectional block and this can be caused by a premature beat. The refractory period of the fast pathway is longer than that of the slow pathway (and also longer than the refractory period of the atrial muscle). In the rabbit, the effective refractory period of the atrial muscle and the fast and slow pathways is  $\sim 81$ ,  $\sim 127$ – $141$  and  $\sim 91$ – $100$  ms, respectively [J. Billette, pers. commun, 37, 38]. Therefore, during a premature beat, conduction along the fast pathway can be blocked and conduction occurs along the slow pathway alone (whereas during the basic beat, conduction can occur along both pathways). Using the voltage-sensitive dye, di-4-ANEPPS, Nikolski et al. [34] mapped the activation sequence of the rabbit AVN region: preparations were paced at the crista terminalis or the interatrial septum. A premature stimulus (S2) was applied after a basic stimulus



**Fig. 7.** AVN reentry in the rabbit. **a** Experiment [34]. A color-coded activation map of conduction during slow/fast AVN reentry is shown superimposed on a photograph of the preparation. **b** Simulation [Inada, unpubl. data]. Isochrones (blue lines) during slow/fast AVN reentry are shown superimposed on the anatomical model. The isochrones are at equal time intervals ( $\sim 5$  ms). The arrows summarize the direction of propagation. In both experiment and model, there is slow anterograde conduction along the slow pathway/posterior nodal extension followed by breakthrough of excitation into the atrial muscle via retrograde conduction along the fast pathway. In the model, the loosely packed atrial muscle (green) was assumed to constitute the fast pathway and is the only connection with the nodal cells next to the enclosed node. A/AN = Atrial-transitional AN tissue; CS = coronary sinus; FP = fast pathway; SP = slow pathway.

(S1). An S1–S2 interval of  $\sim 100$  ms frequently resulted in slow/fast reentry, and figure 7a shows an example of an activation map. Figure 7a shows slow anterograde conduction along the slow pathway followed by breakthrough of excitation into the atrial muscle via retrograde conduction along the fast pathway.

Another function of the AVN is to act as a subsidiary pacemaker in the event of failure of the SAN. Recently, it has been shown that the leading pacemaker site is located in the slow pathway, rather than the enclosed part of the AVN [39].

### *Theoretical Requirements of the Atrioventricular Node for Electrical Coupling*

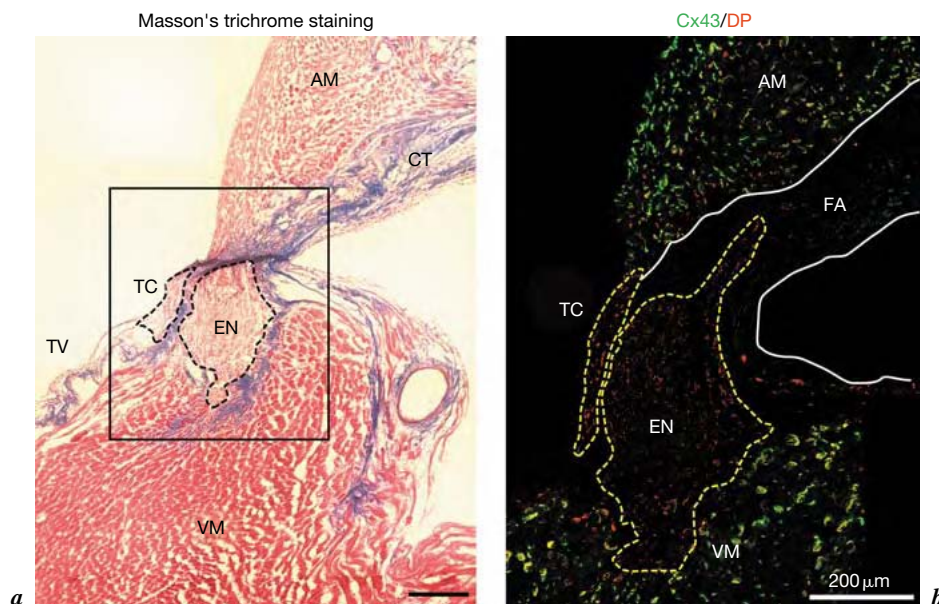
Conduction of the action potential must be slow through the AVN. The conduction velocity is dependent on the upstroke velocity of the action potential as well as the electrical coupling between cells. In the AVN, the upstroke velocity of the action potential is low. In the dog, for example, the upstroke velocity of the action potential is  $\sim 158$ ,  $\sim 60$ ,  $\sim 41$  and  $18$  V/s in the atrial muscle, fast pathway, slow pathway and enclosed part of the AVN, respectively [40]. This is likely to be the result of a variation in the expression of the cardiac  $\text{Na}^+$  channel,  $\text{Na}_v1.5$ : evidence suggests that, whereas  $\text{Na}_v1.5$  is abundantly expressed in the atrial muscle, there is reduced expression of  $\text{Na}_v1.5$  in the fast and slow pathways and no expression of  $\text{Na}_v1.5$  in the enclosed node [41, 42]. In the enclosed node, the action potential upstroke is likely to be supported by the L-type  $\text{Ca}^{2+}$  current,  $I_{\text{Ca,L}}$ , rather than the  $\text{Na}^+$  current,  $I_{\text{Na}}$  [43]. In a simulation study, Shaw and Rudy [44] showed that reducing the density of  $\text{Na}^+$  channels reduces the conduction velocity as expected, but slow conduction via this mechanism alone is not robust, because it occurs with a low safety factor. This mechanism alone cannot support conduction slower than  $10$  cm/s (conduction failure occurs) [44]. Conversely, weak electrical coupling can support slow conduction ( $< 1$  cm/s) with a high safety factor [44]. Interestingly, slow conduction is dependent on  $I_{\text{Ca,L}}$  rather than  $I_{\text{Na}}$ , because depolarizing charge needs to flow through the gap junctions for a relatively long time (to stimulate the upstream cells) and this requires the slow sustained  $I_{\text{Ca,L}}$  rather than the short-lived  $I_{\text{Na}}$  [44]. These arguments suggest that electrical coupling should be weak in the AVN to sustain slow conduction. By analogy with the SAN, electrical coupling in the AVN should also be weak to protect the pacemaking cells of the AVN from the suppressive influence of the nearby atrial cells.

### *Weak Electrical Coupling in the Atrioventricular Node*

The fact that the conduction velocity of the AVN is low is one line of evidence that electrical coupling in the AVN is weak. In an early study, Pollack [45] injected fluorescein into rabbit heart by microiontophoresis and monitored movement of fluorescein into contiguous cells using video microscopy. The rate of passage of fluorescein between the N cells of the compact part of the AVN was at least *three orders of magnitude* less than between other cardiac cells (atrial, ventricular and His-Purkinje cells) [45].

### *Expression of Cx30.2, Cx40, Cx43 and Cx45 in the Atrioventricular Node*

Figure 8b shows immunolabeling of Cx43 in the region of the enclosed node in the rat: whereas Cx43 is abundant in the atrial and ventricular muscle,



**Fig. 8.** Absence of Cx43 in the enclosed part of the AVN of the rat. **a** Masson's trichrome-stained section through the enclosed node. Myocytes are stained red and connective is stained blue. **b** Adjacent section to that shown in **a** double immunolabelled for Cx43 (green signal) and desmoplakin (DP; red signal). Desmoplakin is expressed in desmosomes in all cardiac myocytes. In the enclosed node, Cx43 is not expressed (whereas it is abundantly expressed in the surrounding atrial and ventricular muscle). In contrast, desmoplakin is expressed in the enclosed node as well as the atrial and ventricular muscle (this proves that myocytes are present in the enclosed node). The image in **b** corresponds to the boxed area in **a**. The enclosed node and a zone of transitional cells are ringed with dashed lines. AM = Atrial muscle; CT = connective tissue; EN = enclosed node; FA = fibrous annulus; TC = transitional cells; TV = tricuspid valve; VM = ventricular muscle. Scale bars, 200  $\mu\text{m}$  [Yoo, unpubl. data].

it appears to be absent in the enclosed node (and also in a nearby group of 'transitional cells') [Yoo, unpubl. data, 46]. In the rat and mouse, Cx45 has been reported to be expressed in the AVN and His bundle [47]. In the rat, Cx40 immunofluorescence has been reported in the AVN, but it is of relatively low abundance: the abundance is 10-fold higher in the His bundle and in the Purkinje fibers [48]. In the human, gap junctions in the AVN are reported to be small [18]. They contain Cx45, but they also contain Cx43 and Cx40, although the abundance of Cx43 is low in comparison to that in the working myocardium, and the abundance of Cx40 is low in comparison to that in the His bundle [18]. Kreuzberg et al. [27] reported that Cx30.2 is expressed in the AVN of the mouse. In summary, the complement of gap junction proteins expressed in the AVN

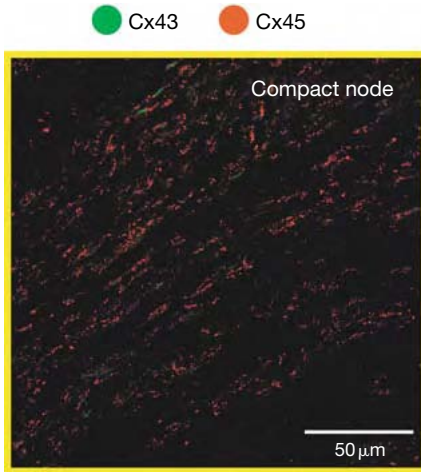


(defined here as the posterior nodal extension and the enclosed node) favors slow conduction: although Cx40 and Cx43, which can form large and medium conductance gap junction channels, can be present in the AVN, Cx30.2 and Cx45, which form small conductance channels, may be the principal connexins.

#### *Model of the Atrioventricular Node*

We have studied the expression of Cx40, Cx43, Cx45 and neurofilament using immunohistochemistry throughout the AVN in the rabbit [39, Li et al., unpubl. data]. One preparation was studied in depth: electrical activity was measured using voltage-sensitive dye and then the preparation was frozen and sectioned and the expression of Cx43 and neurofilament as well as the histology were studied every  $\sim 60\text{--}340\text{ }\mu\text{m}$ . Neurofilament was labeled to identify nodal cells; the cells of the His bundle and enclosed node were, of course, neurofilament positive. The tract of neurofilament-positive nodal cells continued from the enclosed node into the triangle of Koch close to the tricuspid valve. Where this tract projects into the triangle of Koch, it forms the so-called posterior nodal extension. The neurofilament-positive nodal cells adjacent to the enclosed node freely connected with the neurofilament-negative atrial cells (or perhaps transitional AN cells) and this may be the site at which the fast pathway enters the AVN. In contrast, the remainder of the posterior nodal extension was poorly connected or perhaps not connected to the neighboring atrial muscle except perhaps near the termination of the posterior nodal extension below the coronary sinus. The position of the posterior nodal extension as well as its isolation from neighboring atrial muscle (except near its termination) suggests that the posterior nodal extension is the slow pathway. Consistent with this, the measurement of electrical activity showed that the slow pathway (identified as a pathway of slow conduction during reentry) coincided with the posterior nodal extension.

Whereas Cx43 is abundantly expressed in the atrial and ventricular muscle, Cx43 is not expressed in much of the posterior nodal extension. However, Cx43 is sparsely expressed in the enclosed node and His bundle and protruding into the posterior nodal extension from the enclosed node is a tract of cells with sparse expression of Cx43. Whereas Cx45 is little or not expressed in the atrial muscle surrounding the AVN, it is expressed throughout the AVN: throughout the posterior nodal extension, enclosed node and His bundle. As an example, figure 9 from Dobrzynski et al. [39] shows double labeling of Cx43 and Cx45 in the enclosed node of the rabbit: whereas there is little Cx43 labeling, there is abundant Cx45 labeling. In summary, there are two types of nodal cells: nodal cells expressing Cx45 only and nodal cells expressing Cx43 (albeit sparsely) and Cx45. In the case of the preparation studied in depth, we constructed an anatomical model (a mathematical array) of the rabbit AVN (fig. 5b) [49].



**Fig. 9.** Expression of Cx45 in the enclosed part of the AVN of the rabbit. Double labeling of Cx43 (green signal) and Cx45 (red signal) in the enclosed node is shown [39].

In figure 5b, the Cx43-negative/Cx45-positive nodal cells in the posterior nodal extension are shown in red and the Cx43-positive/Cx45-positive nodal cells of the enclosed node are shown in purple. Ko et al. [50] observed a similar pattern of labeling of Cx43 and Cx45 in the rabbit AVN and constructed a similar model. However, they refer to the Cx43-negative/Cx45-positive cells of the posterior nodal extension as ‘transitional cells’, and to the Cx43-positive/Cx45-positive cells of the posterior nodal extension as the ‘posterior nodal extension’, and they suggest that these cells constitute the slow pathway. We believe that it is appropriate to refer to both cell types collectively as the posterior nodal extension, because both cell types express neurofilament (and are, therefore, nodal) and the slow pathway measured in an electrophysiology experiment corresponds to the posterior nodal extension (as defined here). In the rabbit, in the region of the AVN, we have reported Cx40 to be absent in the ventricular muscle, possibly present in the atrial muscle, abundant in the His bundle and present at a low density in the enclosed node and posterior nodal extension [39].

We used our model of the AVN (fig. 5b) to simulate slow/fast reentry (fig. 7b). Once again the cellular automaton model was used to model propagation. The refractory period of the atrial muscle, fast pathway and slow pathway were set to be 81, 134 and 94 ms, respectively. Electrical coupling in the slow pathway was set to be weak ( $R$  was sevenfold less in the AVN than in the atrial muscle), and the conduction velocity of the slow pathway was 2.2 cm/s (whereas the conduction velocity of the atrial muscle was 45.5 cm/s); consequently, conduction along the slow pathway took  $\sim 150$  ms, i.e. a time comparable to the

refractory period. An S2 stimulus was applied 96 ms after the S1 stimulus (model paced at the interatrial septum) and it resulted in slow/fast reentry: figure 7b shows in the model slow antegrade conduction along the slow pathway followed by breakthrough of excitation into the atrial muscle via retrograde conduction along the fast pathway; it is comparable to the experimental result in figure 7a. This illustrates the importance of the weak electrical coupling in the slow pathway in AVNRT. The slow pathway is ablated to abolish AVNRT [35].

We have also investigated the expression of connexins and neurofilament as well as histology at the leading pacemaker site during AVN pacemaking [39]. The leading pacemaker site was shown to be in the posterior nodal extension (as shown by neurofilament expression) and at the site there was little or no expression of Cx43, abundant expression Cx45 and little expression of Cx40 [39]. It is likely that the weak electrical coupling resulting from this pattern of expression of connexins favors pacemaking.

## Conclusion

In conclusion, the SAN and AVN have specialized functions, pacemaking in the case of both nodes and delaying propagation of the action potential from the atria to the ventricles in the case of the AVN. These functions have special requirements as regards electrical coupling and, therefore, expression of connexin isoforms: both pacemaking and slow conduction require weak electrical coupling. Consequently, gap junctions are small and sparse in the nodes and the gap junctions contain little or no Cx40 and Cx43 (responsible for large and medium conductance gap junction channels) and instead they contain Cx45 and Cx30.2 (responsible for small conductance gap junction channels).

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## Cardiac Ischemia and Uncoupling: Gap Junctions in Ischemia and Infarction

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### Abstract

Acute cardiac ischemia is often associated with ventricular arrhythmia and fibrillation. Due to the loss of ATP, the depolarization of the fibers, and the intracellular  $\text{Na}^+$  and  $\text{Ca}^{2+}$  overload with concomitant acidification as well as the accumulation of lysophosphoglyceride and arachidonic acid metabolites, propagation of action potentials will be impaired by two factors: (a) reduced sodium channel availability and (b) gap junction uncoupling. While gap junction uncoupling leads to predominant transverse uncoupling, reduced  $\text{I}_{\text{Na}}$  availability results in impaired longitudinal conduction. Complete gap junction uncoupling would initiate arrhythmia, while intermediate uncoupling has been shown to enhance the safety factor (SF) of propagation, limiting the current loss to non-depolarized areas. In contrast, a reduction in  $\text{I}_{\text{Na}}$  availability reduces SF, and partial gap junction uncoupling might enable effective but slow conduction which, on the other hand, could form the basis for some kind of reentrant arrhythmia, paving the way for new anti-arrhythmic approaches in gap junction coupling. In the chronic phase, remodeling processes also involve gap junctions and lead to highly heterogeneous non-uniform tissue which may serve as an arrhythmogenic trigger.

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Cardiac ischemia and infarction is one of the most common causes of death in Western industrialized countries. Death in these cases is most often caused either by pump failure or by cardiac arrhythmia and ventricular fibrillation (VF). In the past years, it has been suggested that ventricular arrhythmia in these instances (i.e. acute or chronic ischemia/infarction) may be caused by gap junctional uncoupling in the course of acute ischemia or by changes in gap junction protein expression and distribution in the chronic phase of myocardial infarction.

Ischemia acutely leads to loss of high energy phosphates and subsequent breakdown of active processes in the cells. Following the drop in ATP, ATPases

such as  $\text{Na}^+/\text{K}^+$ -ATPase and  $\text{Na}^+/\text{Ca}^{2+}$ -ATPase will stop working, leading to increases in intracellular  $[\text{Na}^+]$  and  $[\text{Ca}^{2+}]$ . Moreover, intracellular acidification will occur, activating  $\text{Na}^+/\text{H}^+$  exchanger and  $\text{HCO}_3^-/\text{Na}^+$  symport, which may partially compensate for intracellular acidosis, however further increasing intracellular  $[\text{Na}^+]$ . As a consequence of these processes, particularly of  $\text{Na}^+/\text{K}^+$ -ATPase failure, the membrane will depolarize. Increases in both intracellular  $[\text{Na}^+]$  and  $[\text{Ca}^{2+}]$  have been described to result in reduced gap junction conductance [1]. High intracellular calcium concentrations (exceeding  $\sim 1 \mu\text{M}$ ) were needed to affect cell coupling with  $\text{pK}_{\text{Ca}}$  values being 6.6, 6.4 and 5.6 at pH 7.4, 7.0 and 6.5, respectively. At each of these pH values, calcium induced uncoupling with a Hill coefficient remaining constant at about 3.4. Noma and Tsuboi [1] concluded from these experiments that  $\text{Ca}^{2+}$  and protons compete for negatively charged binding sites at the  $\text{Ca}^{2+}$  receptor site, being involved in the control of gap junction conductance. They hypothesized that the negative charges necessary for calcium binding may be neutralized by the protons and proposed a cooperative receptor model. While low changes in calcium do not affect gap junction conductance in adult heart cells [2], higher changes in  $[\text{Ca}^{2+}]_i$  reduce gap junction conductance in guinea pig and rat hearts [3]. Maurer and Weingart [3] concluded from their experiments that gap junction conductance decreases if the intracellular calcium concentration exceeds the range of 320–560 nM, which is below the value proposed by Noma and Tsuboi [1]. Maurer and Weingart [3] argued that the difference might be due to different stability constants for the calcium buffer used to calculate the cytosolic  $\text{Ca}^{2+}$  concentration.

In addition, a drop in pH, i.e. an increase in  $[\text{H}^+]_i$ , also leads to a reduction in gap junction intercellular communication (GJIC). Gap junction conductance was nearly constant in a pH range from 7.4 to 6.5 and decreased sharply when pH was reduced to 5.4 [1]. The relationship between pH and gap junction conductance was principally not affected by intracellular pCa. The Hill coefficient was about 2.4, indicating the number of proton binding sites per receptor and a half-maximal concentration of 6.1 ( $\text{pK}_{\text{H}}$ ). In neonatal rat heart cells, Firek and Weingart [4] found a  $\text{pK}_{\text{H}}$  of 5.85. One  $\text{H}^+$  binding site could be identified as histidine-95 in cardiac Cx43 by Ek et al. [5]. Hermans et al. [6] investigated the effects of site-directed mutations in Cx43-transfected SKHep1 cells by exchange of His-126 and His-142 and found an uncoupling effect of acidification related to the position of histidines in the cytoplasmic loop rather than to the total number of histidines. They reported that a fall in  $\text{pH}_i$  caused a reduction in channel open probability but not in channel conductance. Using a transfection system, it was found that Cx45 channels are more sensitive to pH than Cx43 channels [6]. Regarding the pH sensor, the carboxy tail length has been demonstrated as a determinant of pH sensitivity [7]. Further investigations [8]



revealed that the carboxy terminal serves as an independent domain which can bind to another separate domain of the connexin protein (e.g. a region including His-95 [5]) close the channel, comparable to the ball-and-chain model for potassium channels.

Moreover, lysophosphoglycerides and arachidonic acid metabolites accumulate at the intercalated disks and contribute to the reduction in GJIC [9]. Among biochemical alterations, the accumulation of long chain acylcarnitines has been ascribed a role in gap junction uncoupling. Normally, long chain fatty acids can be transported into the mitochondrium by binding to carnitine (via acylcarnitine transferase I), passing the mitochondrial membrane as long chain acylcarnitine. The acyl group is then transferred to intramitochondrial coenzyme A. In the course of ischemia, this mechanism is altered and long chain acylcarnitines accumulate within 2 min after the onset of ischemia *in vivo* [10]. Interestingly, there was a sevenfold accumulation of acylcarnitine in the junctional sarcolemma as compared to the non-junctional regions [9]. Exogenous administration of long chain acylcarnitines resulted in rapid onset of cell-to-cell uncoupling [9]. Inhibition of accumulation of long chain acylcarnitines significantly reduced the incidence of arrhythmia induced by ischemia *in vivo* [11].

Finally, reduced ATP (from 5.0 to 0.5 mM) itself has been described as an uncoupling factor [12] (Hill coefficient: 2.6; half-maximum cytosolic [ATP]: 0.68 mM); reduced [ATP]<sub>i</sub> in ischemic cells will impede phosphorylation while due to ATP shortage dephosphorylation processes will be favored. Dephosphorylation of gap junction proteins was reported to elicit uncoupling [13] (see also the chapter by Hervé and Dhein). Thus, several factors favor gap junctional uncoupling in ischemia. Since electrical coupling is a prerequisite for regular propagation of the action potential and thus for rhythmicity, these considerations suggest a possible role of uncoupling in acute ischemia. On the other hand, it should be noted that this uncoupling may limit the loss of ATP by reducing the electrical and consequently the contractile activity of the cells, as well as by diminishing the drag of ATP from the surrounding non-ischemic tissue into the ischemic region. Thus, uncoupling may, in part, protect from an enlargement in the infarct zone. Experimental evidence supporting this view comes from studies using heptanol as uncoupling agent [14], although it should be noted that heptanol is not specific for gap junction uncoupling. The transfer of 'death factors' would be stopped [15]. On the other hand, 'survival factors' passing via gap junction channels from the non-ischemic zone into the ischemic area will also be hindered [16, 17]. Thus, the factors favoring enlargement of the ischemic zone and those improving survival may counterbalance each other. This view is supported by findings that the ischemic zone was not enhanced in the presence of gap-junction-opening peptides [18] or there were no signs of enhanced ischemic damage [19].

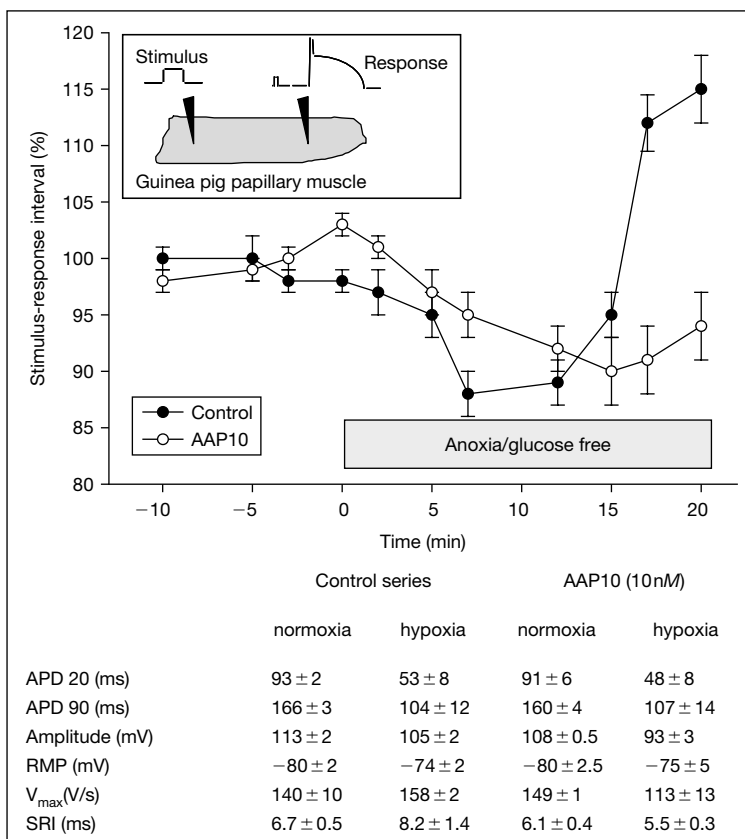
However, closure of gap junctions or reduced GJIC will surely affect propagation of electrical activation, but is this arrhythmogenic? Does it occur during ischemia or infarction? Propagation of the action potential is determined by three factors: (a) sodium channel availability [20], (b) tissue architecture including cell size and shape and interstitial collagen deposition [21] and (c) gap junctions [22]. Gap junctions are made up of connexins, the protein sub-units of these channels. Six connexins form a hemichannel or connexon, and two connexons, one from each of the interacting plasma membranes, constitute the complete channel, which allows the passage of small molecules of up to 1 kDa, e.g. cAMP [23], and the propagation of electrical activation. Macroscopic gap junction conductance ( $g_j$ ) is defined by the number of communicating channels ( $n$ ), the open probability ( $p$ ) and the single channel (unitary) conductance ( $\gamma$ ) according to the equation:

$$g_j = n * \gamma_j * p_{gj},$$

with the unitary conductance differing among the various isoforms of connexins and being subject to regulation by phosphorylation (for review, see Dhein [24] and the chapter by Hervé and Dhein in this book).

## Acute Ischemia

It has been shown by Smith et al. [25] that in acute ischemia there are two peaks of VF: the first appearing within the first 10 min, and the second after about 20 min. Early VF was defined as type Ia VF and late VF as type Ib VF. While type Ia VF has been attributed to factors such as depolarization, mechanical factors and catecholamine release, for example, type Ib VF was suggested to be related to the rise in tissue resistance and uncoupling. Accordingly, de Groot et al. [26] demonstrated an increase in tissue impedance starting 15 min after the onset of ischemia in porcine hearts. Further evidence for uncoupling of the tissue was given by Dekker et al. [27] in a perfused rabbit papillary muscle showing an abrupt rise in tissue resistance about 12 min after the onset of ischemia. In a similar approach in guinea pig papillary muscle, we also demonstrated a reduction in conduction velocity 15 min after simulated ischemia (deep hypoxia with glucose-free superfusion; fig. 1). We found slight depolarization and action potential shortening, and after >15 min a significant increase in conduction time accompanied by increased  $dU/dt_{\max}$  [28]. This may reflect uncoupling since the increase in  $dU/dt_{\max}$  may be due to reduced current loss due to non-depolarized cells. However, some reduction in sodium channel availability following depolarization will probably also contribute to the observed slowing. However, in agreement with the above studies, 12–20 min after the onset

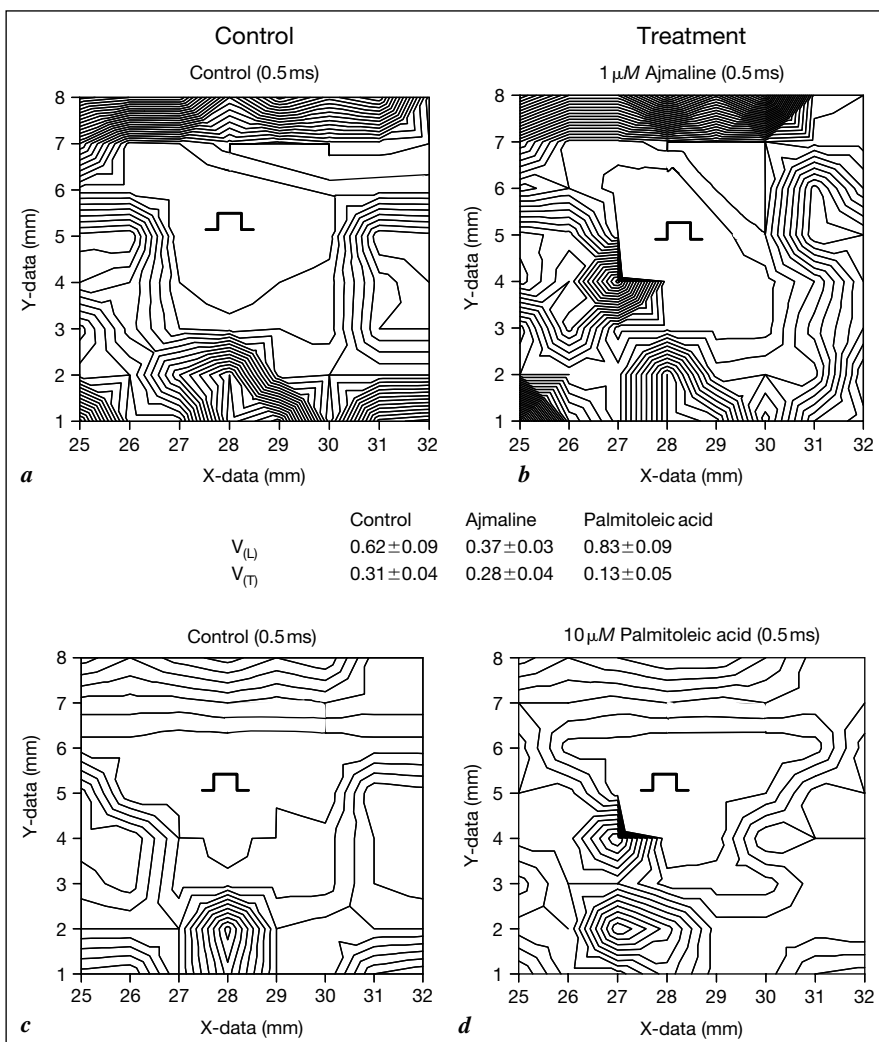


**Fig. 1.** Stimulus-response time, i.e. conduction delay between the stimulation site at the base of a guinea pig papillary muscle and the tip of the muscle, where the propagated action potential was recorded, is shown. The inset demonstrates the setup. After an equilibration period under standard conditions, simulated ischemia was induced by superfusion with glucose-free Tyrode solution at low oxygen tension ( $pO_2 \sim 20$  mm Hg). The data of the action potentials recorded with and without additional treatment with the anti-arrhythmic peptide AAP10 (10 nM) are given below. APD = Action potential duration; RMP = resting membrane potential; SRI = stimulus-response interval.

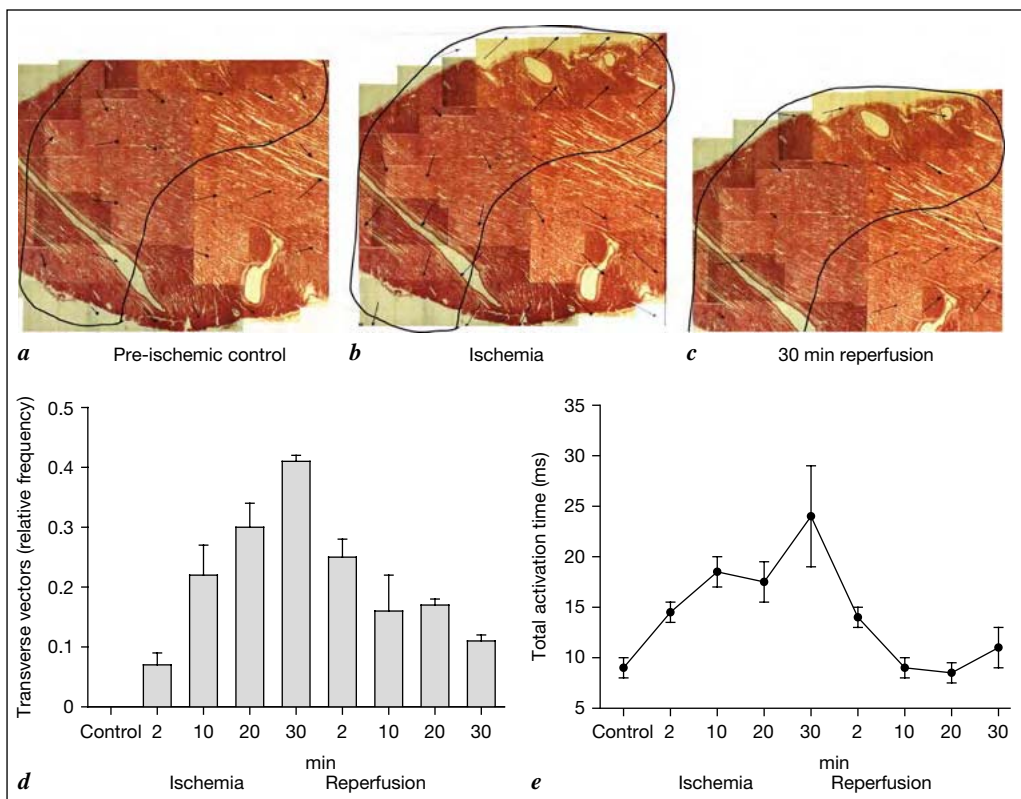
of ischemia, conduction decreased and uncoupling occurred, possibly coinciding with the peak in type Ib VF. The question arises whether both observations are pathophysiologically linked to each other. In order to address this question, it is necessary to compare the effects of a gap junction blockade to those of an  $I_{Na}$  blockade. Therefore, we used a 256-channel epicardial potential mapping system (HAL4; Peter Rutten, Hamburg, Germany, described previously [29, 30]) for mapping of the activation propagation along and transverse to the fiber

axis in isolated, spontaneously beating rabbit hearts (New Zealand White rabbits, body weight: 2 kg, Charles River) perfused with Tyrode solution (composition:  $\text{Na}^+$  161.02,  $\text{K}^+$  5.36,  $\text{Ca}^{2+}$  1.8,  $\text{Mg}^{2+}$  1.05,  $\text{Cl}^-$  147.86,  $\text{HCO}_3^-$  23.8,  $\text{PO}_4^{2-}$  0.42 and glucose 11.1 mM, equilibrated with 95%  $\text{O}_2$  and 5%  $\text{CO}_2$ , pH = 7.4) at a constant pressure of 70 cm  $\text{H}_2\text{O}$  according to the Langendorff technique. Activation times were determined as  $t(\text{dU}/\text{dt}_{\min})$  [31]. At the back wall, action potentials were propagated by application of rectangular pulses (1-ms duration, double threshold), and the activation patterns were mapped under standard control conditions after 45 min of equilibration and after intracoronary infusion of either 10  $\mu\text{M}$  palmitoleic acid ( $n = 6$ ), a substance inducing gap junction uncoupling [32, 33], or 1  $\mu\text{M}$  ajmaline ( $n = 3$ ), a sodium channel blocker, both treatments being followed by a 15-min washout. We found that ajmaline predominantly reduced longitudinal conduction velocity ( $V_L$ ) while palmitoleic acid led to a decrease in transverse ( $V_T$ ) rather than  $V_L$  (fig. 2), in agreement with a previous study showing predominant transverse uncoupling under palmitoleic acid [33]. This drug has been shown to uncouple gap junctions at concentrations  $>2 \mu\text{M}$  [32], with an  $\text{EC}_{50}$  of about 5  $\mu\text{M}$  [33]. This concentration range (2–10  $\mu\text{M}$ ) does not affect action potential duration, amplitude or resting membrane potential [33]. A predominant transverse uncoupling was also observed with the uncoupling agent heptanol [34]. Thus, gap junction uncoupling predominantly reduces transverse conduction (this has been suggested to be due to the fact that there are only a few gap junctions at the lateral border of the cells enabling transverse propagation which might thus be more sensitive to effects at these few gap junctions), while  $I_{\text{Na}}$  blockade or reduced  $I_{\text{Na}}$  availability mainly affect  $V_L$  [20]. In consequence, anisotropy ( $V_L/V_T$ ) is enhanced by gap junction blockade and reduced by  $I_{\text{Na}}$  blockade.

Since ischemia may reduce GJIC by a number of factors and, on the other hand, will also lessen  $I_{\text{Na}}$  availability due to depolarization, the question arises how propagation will change during ischemia. To answer this question, 256-channel epicardial potential mapping was performed on spontaneously beating, isolated rabbit hearts (New Zealand White rabbits, body weight: 2 kg; Charles River) perfused with Tyrode solution (composition as described above) at constant pressure (70 cm  $\text{H}_2\text{O}$ ) according to the Langendorff technique [30]. After 45 min of equilibration under standard conditions, we induced local ischemia by ligation of a branch of the left anterior descending coronary artery for 30 min followed by release of occlusion and 30-min reperfusion. Activation times were determined as described above. For each electrode, a vector giving direction and apparent velocity of local activation was calculated from the activation times and the locations of the surrounding electrodes, which were activated after the central electrode (i.e. a maximum number of 8), as described by Müller et al. [35] and Dhein [30]. The resulting vectors were projected on the histological sections,



**Fig. 2.** Isochrones (0.5 ms) of electrical activation around the stimulation site at the back wall of an isolated rabbit heart. A ventricular response was elicited with a rectangular pulse (1 ms, double threshold) at the site indicated and the activation was mapped as described. After equilibration (control, **a**, **c**) either 1  $\mu M$  ajmaline (**b**) or 10  $\mu M$  palmitoleic acid (**d**) were administered by intracoronary infusion. Mean  $V_{(T)}$  and  $V_{(L)}$  values are also listed (means  $\pm$  SEM of  $n$  experiments).



**Fig. 3.** Vectors of epicardial activation during pre-ischemic control (*a*), during 30 min of ischemia (*b*) and at the end of the 30-min reperfusion (*c*) projected on the histological sections. Note the change in direction during ischemia. The ischemic area is encircled. The number of transverse vectors (*d*,  $n = 6$  experiments) and the total activation time (delay between activation of the first and the last electrode, *e*) are also shown (means  $\pm$  SEM).

and the angle between vector and local longitudinal fiber axis was measured. Next we calculated the number of vectors deviating  $65\text{--}90^\circ$  from the longitudinal fiber axis and defined them as transverse vectors. We found that the number of these vectors increased during ischemia indicating a turn of the vector field to the transverse direction. Upon reperfusion, the vectors returned to their original direction (fig. 3a–d). Concomitantly, general slowing of conduction was evidenced by prolongation of total activation time (defined as the delay between activation of the first and the last electrode [30]; fig. 3e). Is, according to the above considerations, this turn of the vector field to the transverse direction against the hypothesis of ischemic uncoupling, since gap junction uncoupling was associated with predominant slowing in transverse conduction? To interpret

these results we have to consider that via ATP loss ischemia leads to depolarization which should result in reduced  $V_L$  as well as to  $[Na^+]_i$  and  $[Ca^{2+}]_i$  increases accompanied by reduced pH, which combined effect gap junction uncoupling thereby probably attenuating  $V_T$ . However, cell size and shape also determine the direction of propagation and modulate the effect of gap junction uncoupling and reduced  $I_{Na}$  availability on the direction of propagation [21, 22]. Thus, with respect to the tissue, the main triggers of conduction are found along the fibers and along the plasma membrane thus depending on  $I_{Na}$  availability rather than on gap junction coupling. This would be in good agreement with the finding of a turn of the vector field to the transverse direction during ischemia (fig. 3).

However, there are several important arguments against a role of uncoupling: Jongsma and Wilders [36] demonstrated that a 90% decrease in the number of gap junctions is required to reduce conduction velocity by only 25%. However, this refers to normoxic standard conditions. In line with these considerations, heterozygous Cx43 knockout mice exhibited only a small effect on conduction, with about 25% reduction [37–39]. In addition, in simulated ischemia in cell pairs, gap junction coupling remained large enough to equilibrate action potential duration between the coupled cells until inexcitability occurred [40].

In contrast, the number of hearts exhibiting pacing-induced VT in Cx43+/+ or in Cx43+/- mice was higher in the group of Cx43+/- mice (11/15) compared to the Cx43+/+ population (4/15) [38], which is in favor of an arrhythmogenic role of gap junctions (or at least Cx43). In support of this, we could demonstrate that a peptide (AAP10,  $H_2N$ -Gly-Ala-Gly-Pro-[4-OH-Pro]-Tyr-CONH<sub>2</sub>) which enhanced macroscopic gap junction conductance [28, 41, 42] without affecting the action potential, could prevent late ischemic type Ib VF in isolated rabbit hearts submitted to 40 min of ischemia (occlusion of the left anterior descending coronary artery) at 2.5 mM  $K^+$  [19]. Similar anti-arrhythmic effects could also be shown by another group using a chemical derivative of AAP10, i.e. ZP123 [18], supporting our previous findings with AAP10. Moreover, in guinea pig papillary muscles subjected to simulated ischemia (deep hypoxia and glucose-free superfusion), 10 nM AAP10 prevented uncoupling. Interestingly, while normally  $dU/dt_{max}$  was increased in simulated ischemia, this parameter was reduced if the peptide was applied (fig. 1). This can be explained by the assumption that under these conditions opening of gap junctions will probably allow current to flow from the excited cells to the non-depolarized cells thereby decreasing  $dU/dt_{max}$ , while closure of gap junctions under simulated ischemia should have the opposite effect [28]. However, one might argue that at certain (probably small) degrees of keeping gap junctions open, such prevention from uncoupling can prevent from arrhythmia, but at higher degrees opening of gap junctions might favor current flow from the excited cells to the surrounding cells thereby diminishing the amount of local current load necessary for local activation and thus possibly leading to conduction failure. However,

it should be noted that we did not observe conduction failure with AAP10 in any of our experiments including ischemia/reperfusion experiments, possibly since the AAP10 effect is moderate.

In ischemia, both gap junction coupling and  $I_{Na}$  availability are reduced simultaneously, which both affect the safety factor (SF) of propagation. SF is defined by the quotient of charge produced by a given membrane patch during activation divided by the charge consumed during the activation process. As long as SF is larger than 1.0, conduction will be possible; as SF drops below 1.0 conduction will fail [43, 44]. In computer simulations, a reduction in  $I_{Na}$  conductance from 100 to 10% leads to decreased conduction velocity and finally failure as conduction velocity approaches 17 cm/s from initially 54 cm/s, while SF is concomitantly reduced from 1.6 to 1.0 and  $dU/dt_{max}$  decreases from about 240 to 25 V/s [44]. In the same model, progressive decrease in intercellular coupling from 3 to 0.008  $\mu S$  also led to a drop in conduction velocity, but up to 0.01  $\mu S$  SF increased (!) concomitant with  $dU/dt_{max}$ . Below 0.009  $\mu S$ , decreases in SF and  $dU/dt_{max}$  and conduction failure were observed. The increase in  $dU/dt_{max}$  and SF can be explained by a diminished current loss to non-depolarized cells. Thus, if gap junction uncoupling occurs in concert with reduced  $I_{Na}$  conductance as in acute ischemia, closure of gap junctions would limit current shunting downstream and enhance availability of inward current for local depolarization. Therefore, gap junction uncoupling may partially compensate for reduced SF (due to reduced  $I_{Na}$  availability) preserving slow but still effective conduction. Under these ischemic conditions, even smaller changes in gap junction conductance may affect the biophysics of propagation, while under normal conditions gap junction conductance probably exerts a lower effect on conduction velocity. An important point to mention in this discussion is the study by Rohr et al. [45] showing that the balance between current source and sink (= area to which current is flowing from the activated site) is important for successful conduction. In this regard, enhanced gap junction coupling may even cause conduction failure if the current source is small and the sink is large, or – vice versa – reduced gap junction coupling may limit current loss, thus keeping  $SF > 1.0$  and providing effective conduction. However, this should not be simply generalized since the local conditions and architecture, and the resulting current source/sink ratios have to be taken into account.

Finally, uncoupling of gap junctions in ischemia is a double-edged phenomenon: on the positive side it may preserve effective conduction under ischemic conditions, and, thus, may preserve rhythm under certain conditions, and on the negative side uncoupling may change the activation pattern and thereby act arrhythmogenic. The slow but still effective conduction may form the basis for arrhythmia usually considered to be linked to slow conduction, e.g. some kind of reentrant arrhythmia. In consequence, improvement in gap junction coupling



may act anti-arrhythmically on certain types of arrhythmia, e.g. type Ib VF, but on the other hand may favor others by unmasking regions of unidirectional block. However, besides simply antagonizing uncoupling-induced conduction failure, the improvement in gap junction coupling may also (hypothetically) enhance current loss thereby leading to conduction failure of the still preserved slow ischemic conduction, which may form the basis for reentrant circuits, so that improved gap junction coupling could also act anti-arrhythmically by this complex mechanism. Future studies will have to elucidate this hypothesis.

### **Chronic Ischemia/Infarction**

In survivors of the acute phase of ischemia, blood supply is not completely restored, and prolonged hypoxia, tissue necrosis, infarction, and remodeling will occur, and the patients' life is still threatened by arrhythmia.

In the chronic phase of myocardial ischemia, remodeling processes start with an increase in fibrosis and changes in the surviving myocardium. Among these changes (a detailed review of all the processes is beyond the scope of this article), remodeling of gap junctions with a general decrease in Cx43 and changes in the distribution of Cx43 and Cx40 occurs (more details are given in the chapter by Severs et al. in this book). Following infarction, local contractility changes and the necrosis zone is replaced by connective tissue. Fibroblast growth factor (FGF-2), which can be released from cardiomyocytes during contraction and after stimulation with catecholamines, is upregulated in response to myocardial damage [46, 47] and can decrease intercellular dye coupling. It induces Cx43 phosphorylation on serine residues, tyrosine phosphorylation and a masking of Cx43 epitopes in cardiomyocytes, whereas in fibroblasts coupling was found to be increased in response to FGF-2 [46, 47]. However, at present, it is unclear whether this factor affects adult and neonatal cardiomyocytes, which have been examined, to the same extent. It is tempting to speculate that these changes might be somehow linked to arrhythmias observed after myocardial infarction originating from abnormal conduction of activation in the vicinity of scar tissue [48]. Slowed anisotropic conduction was found to surpass the margins of the infarct [49].

Interestingly, only *subacute* hypoxia without simultaneous shortage in glucose supply is able to initiate a gap junction remodeling process in cultured neonatal rat cardiomyocytes with a decrease in total Cx43 by 50%, while the non-phosphorylated form was unchanged [50]. Both gap junction number and size were found to be decreased within 5 h of hypoxia and conduction velocity was concomitantly reduced by about 20%.

The effects seen in the *chronic* phase after infarction are: (a) loss of the common ordered (polarized) distribution of the gap junctions, which was found

predominantly in the border zone adjacent to infarct scars, and (b) reduction in the quantity of Cx43 gap junctions in areas distant from the infarct zone [51] (for details see the chapter by Severs et al.). This and other factors may result in a heterogeneous anisotropic conduction and locally reduced conduction velocity forming a pro-arrhythmic substrate. The active properties of cells and resting membrane potential can be quite normal in the presence of manifest cardiac arrhythmia, indicating that passive electrical properties may be of importance [49, 52, 53]. In light of the present research, gap junctions are one of the most important determinants of these passive conduction properties [48, 54]. In the hearts of patients with end-stage ischemic heart disease and in biopsies from patients 3 months after myocardial infarction, gap junction distribution in histologically normal areas was almost normal. In contrast, within the border zone of healed infarcts (some hundred micrometers from the infarct scar) the pattern of gap junction distribution is disturbed, with gap junctions being scattered over the whole cell surface instead of being confined to the intercalated disks at the cell poles. These border zone myocytes also exhibit substantial heterogeneity with regard to orientation and ultrastructure and sometimes a disorganization of the intercalated disks. Myocytes were observed which communicated via cell processes with gap junctions in the absence of fasciae adherentes. In addition, some annular gap junction profiles indicated possible internalization of gap junctions. Normal cells also occur between all these cells [51]. Among all these cell types, fibroblast-myocyte coupling probably plays a special pathophysiological role (for details on this interesting phenomenon, see the chapter by Camelliti et al.). Interestingly, successful conduction can be established between cardio-myocytes separated by (non-excitabile) fibroblasts over a distance of up to 300  $\mu\text{m}$  [55]. Thus, decreased Cx43 values, alterations in their distribution, decreased in gap junction numbers and alterations in gap junction size, remodeling processes, fibroblast-myocyte coupling all together initiate a complete change in the functional architecture of the tissue leading to high heterogeneity and non-uniform anisotropy with the formation of an arrhythmogenic substrate and high degree of non-uniformity.

Novel therapeutic approaches will have to be focussed to influence this remodeling process and the fibrotic tissue.

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## Connexin43 and Ischemic Preconditioning

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### Abstract

Connexin43 (Cx43) is the essential protein to form hemichannels and gap junctions in the myocardium. The phosphorylation status of Cx43 which is regulated by a variety of protein kinases and phosphatases determines hemichannel and/or gap junction conductance and permeability. Gap junctions are involved in cell-cell coupling while hemichannels contribute to cardiomyocyte volume regulation. Cx43-formed channels are involved in ischemia/reperfusion injury, since blockade of a large portion of Cx43-formed channels attenuates ischemic hypercontracture, infarct development and post myocardial infarction remodeling. Ischemic preconditioning's protection also depends on functional Cx43-formed channels, since uncoupling of channels or genetic Cx43 deficiency abolishes infarct size reduction by ischemic preconditioning. The exact underlying mechanism(s) how Cx43 mediates protection remain to be established.

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In the heart, gap junctions play an essential role for normal contractile function, since they facilitate major ionic fluxes between adjacent cardiomyocytes, thereby allowing electrical synchronization of contraction. Each single gap junction is composed of 12 connexin 43 (Cx43) units, assembled in two hexameric connexons (hemichannels) which enable the connection of two neighboring cells [1]. Most gap junctions are located at the terminal intercalated disks of the cell, but they also exist at the lateral sarcolemma. A transmural gradient of Cx43 and thus gap junction expression exist across the left ventricular wall [2, 3], with a higher expression in the endocardium than in the epicardium [4]. Gap junctions propagate the electrical activity in the longitudinal and transverse direction [3],

and conductance in pairs of mouse myocytes is similar in end-to-end (longitudinal) or side-to-side (transversal) connections [5]. Some unopposed connexons are also located at the lateral sarcolemma and connect the intracellular and extracellular space. Until recently, these so-called hemichannels were thought to remain permanently closed in order to avoid cell death [6]; new data, however, have documented the existence of regulated hemichannel opening in cultured cells [7]. Hemichannels appear to be involved in cellular responses such as the release of cytosolic components, e.g.  $\text{NAD}^+$  and ATP [8], activation of cell survival pathways [9] and volume regulation [10].

Cx43 has four transmembrane, two extracellular and three cytosolic (including the amino and carboxy terminus) domains; residues 1–242 form the plasma channel portion, and residues 243–382 the cytosolic tail of Cx43 [11]. The length of the cytosolic tail of Cx43 slightly varies between different species and tissues [12].

Connexins interact with other proteins within the cell. Recent studies suggest an association between Cx43 and the peripheral membrane zona occludens (ZO)-1 protein in rat cardiomyocytes [13]. ZO-1, in turn, binds to  $\alpha$ -spectrin, a protein highly expressed at the intercalated disk [13, 14]. Furthermore, Cx43 co-localizes with fibroblast growth factor receptors [15] and cytoskeletal proteins [7, 16].

### **Regulation of Hemichannels and Gap Junctions**

Cx43-formed hemichannels are not ion selective [17] and permeable to organic ions and molecules of a molecular weight  $<1$  kDa and a maximal diameter of  $\sim 1.5$  nm. The transport of ions and small molecules through hemichannels and gap junctions is mediated by passive diffusion and thus depends on the concentration difference between connected cells and the electrical charge of the moving ions or molecules. The transfer of ions and small molecules further depends on the number of available channels (assembly), their open probability [18] and the individual channel conductance. The number of available channels depends on the synthesis, transport, half-life and breakdown of Cx43 [7].

Cx43-formed gap junctions exhibit three conductance states: one of 20–30 pS, one of 40–60 pS and one of 70–100 pS [1, 19, 20]. The conductance of Cx43-formed hemichannels ranges from 31 to 352 pS [21]. More recently, the conductance of fully open hemichannels in HeLa-cells was found to be 220 pS; however, a substate of approximately 75 pS also exists between the fully open and the closed state [22], suggesting that hemichannel conductance can be regulated as well.

On Western blot, Cx43 exhibits three bands with molecular weights ranging from 41 to 46 kDa depending on the number and epitopes being phosphorylated [23–26].

Single channel conductance as well as cell-to-cell conductance and permeability can be modulated by the intracellular pH, the intracellular calcium or ATP concentrations and the phosphorylation status of Cx43 [for details, see ref. 27–29]. However, single channel conductance and cell-to-cell conductance can be affected in the opposite direction. Cell-to-cell conductance in the presence of decreased single channel conductance might nevertheless be increased by increased single channel open probability or increased channel assembly. Furthermore, regulatory mechanisms can have opposite effects on cell-to-cell electrical coupling and dye transfer if they have divergent effects on single channel open probability and channel pore size [30, 31]. Finally, regulatory mechanisms interact and depend on the prevailing circumstances, since acidosis in normal hearts reduces dye transfer between cardiomyocytes, while the same level of acidosis during ischemia does not [32]. Protein kinases (PKs) and phosphatases (PPs) which are involved in the regulation of single channel conductance and cell-to-cell conductance and permeability are shown in figure 1.

#### *Protein Kinase A*

Cyclic adenosine monophosphate (cAMP) activates PK A (PKA), which in turn phosphorylates Cx43 in rat cardiomyocytes [33]. Increases in the cAMP concentration increase electrical conductance between paired cardiomyocytes [31, 34, 35] and cell permeability, assessed as dye transfer, in non-cardiomyocytes [36–38]. Apart from increased cell-to-cell conductance and permeability, cAMP also increases the extent of gap junction formation [36–38]. Increased cAMP concentration results from its enhanced production following stimulation of adenylyl cyclase or from inhibition of phosphodiesterase III secondary to an increased concentration of cyclic guanosine monophosphate (cGMP) [39–41].

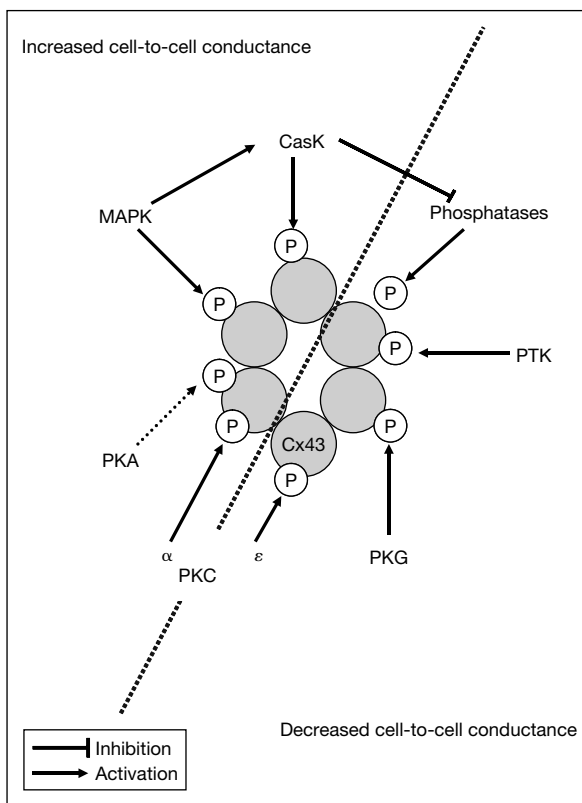
#### *Cyclic-Guanosine-Monophosphate-Dependent Protein Kinases*

At a higher concentration, cGMP activates PK G (PKG) [for review, see ref. 40]. cGMP decreases single channel conductance in rat cardiomyocytes [20, 31] as well as cell-to-cell conductance [20, 31, 34] and permeability [31].

#### *Protein Kinase C*

PK C (PKC) isoforms ( $\alpha$  and  $\epsilon$ ) form signaling complexes with Cx43 [42, 43] and phosphorylate Cx43 in cardiomyocytes [44–46], resulting in decreased single channel conductance and cell-to-cell permeability [30, 31]. On the other





**Fig. 1.** Schematic diagram of PPs and PKs on opening or closure of connexons. CasK = Casein kinase; P = phosphorylation.

hand, electrical conductance between paired cardiomyocytes is increased following PKC activation [30, 31, 47], which could be explained by differences in the single channel open probability and pore size following channel phosphorylation.

### *Protein Tyrosine Kinase*

Protein tyrosine kinase (PTK), e.g. src kinase, phosphorylates tyrosine residues 247 and 265 of Cx43. In paired rat cardiomyocytes, permeability is reduced with phosphorylation of Cx43 by PTK [44, 48]. Phosphorylation of Cx43 at tyrosine residue 265 reduces the binding of Cx43 to ZO-1 and subsequently the expression of Cx43 at the intercalated disks [49]. Reduced intercellular communication following lipopolysaccharide administration is related to Cx43 phosphorylation at tyrosine residues [50].

### *Mitogen-Activated Protein Kinases*

More recently, mitogen-activated PKs (MAPKs) such as erk [51–54], BMK-1 [55], p38 [43, 51] and jnk [56, 57] have been implicated in the regulation of Cx43 phosphorylation. BMK-1 phosphorylates the serine residue 255 of Cx43 [55], while other MAPKs contribute to Cx43 phosphorylation of serine residues 279 and 282 [54, 58, 59]. Increased phosphorylation of Cx43 by MAPKs increases electrical conductance of paired rat cardiomyocytes [51].

### *Casein Kinase*

Casein kinase 1 contributes to Cx43 phosphorylation at serine residues 325 and 330. Such increased Cx43 phosphorylation leads to increased non-junctional membrane expression of Cx43 and decreased cell-to-cell conductance in rat kidney cells [60].

### *Protein Phosphatases*

PPs colocalize with Cx43 in porcine cardiomyocytes [61] and contribute to Cx43 dephosphorylation [62, 63], and dephosphorylation of Cx43 increases single channel conductance in rat cardiomyocytes [20].

Apart from the fact that different PKs and PP independently contribute to the regulation of single channel conductance and cell-to-cell conductance and permeability, there exists also a substantial cross-talk between the different PK/PP pathways. For example, cGMP not only activates PKG but also activates p38 MAPK, which, in turn, can induce PP translocation from the cytosol to the membrane [64]. Similarly, activation of PKC and/or PTK can activate MAPKs [65] which subsequently may induce activation of casein kinase [66], thereby inhibiting PP [67]. Activation of PP, in turn, dephosphorylates MAPKs, thereby decreasing their activities [68].

### *Proton and Calcium Concentration*

Apart from activation of PKs, increases in the intracellular proton and calcium concentrations or a decrease in the intracellular ATP concentration gradually decrease cell-to-cell conductance [for review, see ref. 27]. However, while acidosis in normal hearts reduces dye transfer between cardiomyocytes, the same level of acidosis during ischemia does not [32], pointing to an interaction of several of the above regulatory mechanisms.

## **Myocardial Ischemia/Reperfusion Injury and Its Modification by Ischemic Preconditioning**

Cardiomyocyte death occurs during ischemia as well as during the subsequent reperfusion [69], with both necrosis and apoptosis contributing to

cell death [70]. Loss of cardiomyocyte volume regulation contributes to irreversible ischemic tissue injury [71], and open hemichannels might contribute to the ischemia/reperfusion-induced osmotic imbalance in cardiomyocytes [72, 73].

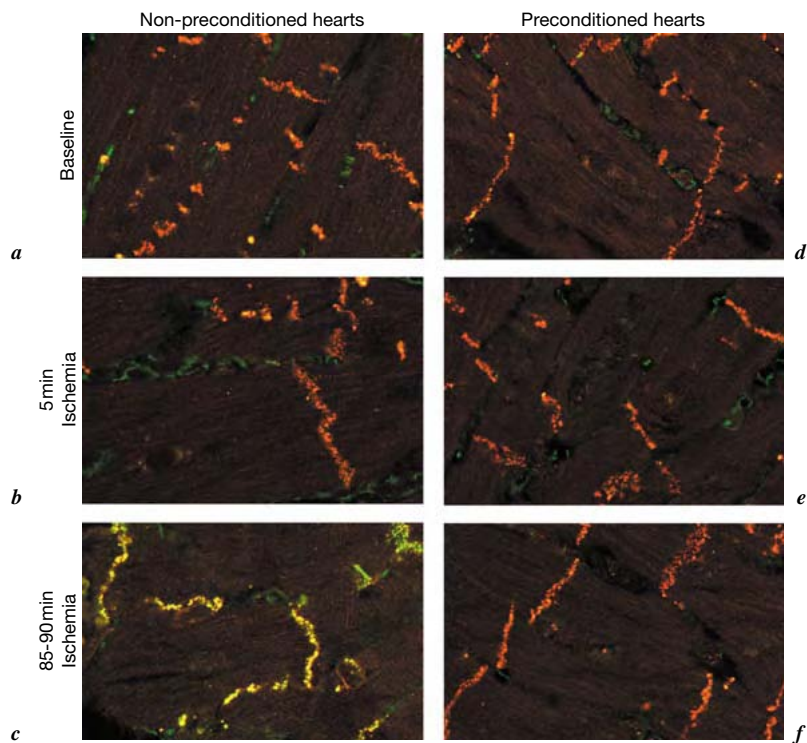
Brief episodes of ischemia/reperfusion delay the development of irreversible tissue damage induced by a subsequent more prolonged ischemic period [74]. Apart from the delay in infarct development, ischemic preconditioning also reduces the extent of apoptosis [75–77]. The signal transduction cascade of ischemic preconditioning has been discussed in detail elsewhere [71, 78].

### **Alterations in Connexin 43 during Ischemia**

In normally perfused myocardium, most of the Cx43 is in a partially phosphorylated state, and it remains in a phosphorylated state within the first minutes of ischemia [43]. With prolongation of ischemia, however, Cx43 becomes dephosphorylated [43, 79–81] (fig. 2), most likely due to an unaltered or increased activity of PP and a reduction in the energy available for PKs. The time course of progressive Cx43 dephosphorylation is closely related to that of electrical uncoupling in isolated rat hearts [79, 80]. However, although ischemia clearly impairs electrical cell coupling, cell-to-cell permeability in general (assessed by dye transfer) cannot be easily deduced from electrophysiological observations [82]. Indeed, in isolated rat hearts electrical coupling between cardiomyocytes is reduced following 10 min of global ischemia, while dye transfer between cardiomyocytes persists for up to 45 min of global ischemia [32]. In mouse astrocytes, simulated ischemia reduces gap junction communication between cells, but even induces opening of non-junctional hemichannels [6]. In isolated cardiomyocytes, simulated ischemia causes opening of hemichannels, too [72, 73]. Opening of hemichannels contributes to the elevation in intracellular sodium and calcium concentrations during simulated ischemia in rabbit ventricular cardiomyocytes [72].

With a single channel conductance of more than 100 pS [1, 20, 22], only ten hemichannels need to be open to produce a millimolar cellular sodium influx [83], and such a millimolar increase in the intracellular sodium concentration has been measured during ischemia in whole hearts [84–86]. The osmotic imbalance resulting from increased AMP, inorganic phosphate and sodium concentrations results in swelling and finally membrane rupture of the ischemic cells.

In addition, an increased intracellular calcium concentration in the presence of some remaining or restored energy production can induce cardiomyocyte



**Fig. 2.** Representative examples of total Cx43 (red) and non-phosphorylated Cx43 (green) from a non-preconditioned heart (**a-c**) and a preconditioned heart (**d-f**). While in non-preconditioned hearts the density of non-phosphorylated Cx43 at the intercalated disks increased during 85–90 min of ischemia (yellow color, **c**), the density remained unchanged in preconditioned hearts (**f**).

hypercontracture [69], and opening of gap junctions might be involved in the transmission of factors triggering hypercontracture between adjacent cells [32, 87], such as sodium for example [87, 88]. Indeed, cell-to-cell transmission of hypercontracture can be attenuated in isolated rat cardiomyocytes, in rat hearts in vitro and in pig hearts in vivo by the gap junction uncoupler heptanol (1–2 mM); in pig hearts in vivo heptanol reduces both myocardial shrinkage and infarct size [89]. Similar results have been obtained with butanedione monoxime, another gap junction uncoupler [90], in pig hearts in vivo [91].

Thus, early during ischemia, gap junctions contribute to the transmission of factors triggering hypercontracture. With prolongation of ischemia, modification of gap junction conductance by dephosphorylation of Cx43 induces electrical uncoupling. Although ion transfer through gap junctions might be impaired, the

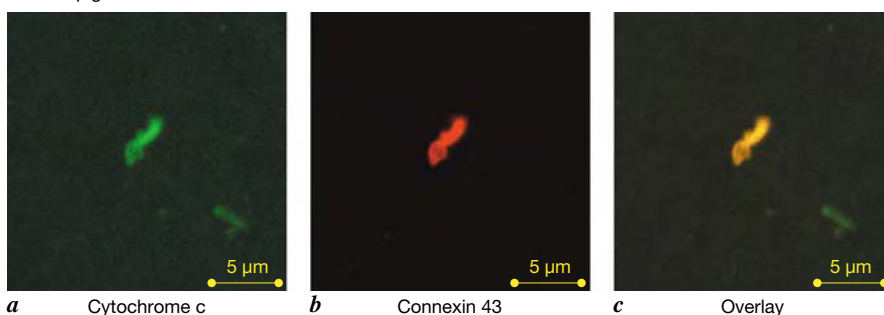
transfer of other small molecules (cell-to-cell permeability in general) remains unaltered. Regulation of hemichannels and gap junctions during ischemia can differ, and opening of hemichannels during simulated ischemia in isolated cells contributes to cell swelling and induction of irreversible tissue injury.

### *Connexin 43 and Ischemic Preconditioning*

Both changes in the electrical coupling between cardiomyocytes and in the channel permeability have been demonstrated following ischemic preconditioning. Cx43 dephosphorylation is decreased in preconditioned compared to non-preconditioned myocardium [43, 80], and the electrical uncoupling, which is closely related to Cx43 dephosphorylation [79], is almost completely abolished by ischemic preconditioning in rat hearts [80]. Decreased channel permeability could protect cardiomyocytes against sodium and subsequently volume overload, and indeed cardiomyocytes become more resistant towards a hypotonic challenge once they are preconditioned [92]. Administration of fibroblast growth factor-2, which is known to reduce cell-to-cell permeability in cardiomyocytes via PTK activation and Cx43 phosphorylation [44, 48], mimics the protective effect of ischemic preconditioning in rats [93] and pigs [94, for review, see ref. 95]. Finally, even the passage of a 'death factor' [82, 96] during the sustained ischemia between adjacent cells could be reduced in preconditioned hearts with reduced cell-to-cell permeability.

An alternative explanation of the protection afforded by Cx43 relates to the preconditioning ischemic period per se rather than the sustained ischemic episode. During the preconditioning ischemia, a 'survival factor' [97] could pass through severely ischemic cardiomyocytes via connexons, thereby placing connected cells or cells in close proximity into a protected state. Factors shown to be released from cardiomyocytes that can induce a preconditioning phenomenon in cardiomyocytes are calcium ('calcium preconditioning') and adenosine [for review, see ref. 71]. Indeed, uncoupling of connexons with heptanol in mice [97] or genetic Cx43 deficiency [98, 99] abolishes protection induced by ischemic preconditioning.

It remains to be determined whether the effect of Cx43 on the protection induced by ischemic preconditioning relates to alterations in gap junction communication or to changes in volume homeostasis. An in vivo study in porcine hearts [100] found protection but no alteration in ischemia-induced changes in cardiac impedance and therefore favored a non-gap-junction-mediated mechanism. In a previous study, the protective effect of ischemic preconditioning was also abolished in isolated cardiomyocytes from heterozygous Cx43-deficient mice, i.e. in the absence of cell-to-cell communication via gap junctions [101].



**Fig. 3.** Purified pig left ventricular mitochondria were stained with antibodies against cytochrome c (green, **a**) and Cx43 (red, **b**) and analyzed by confocal laser scan microscopy. **c** Merged image shows colocalization pixels in yellow.

Although it is generally assumed that Cx43 is confined to the sarcolemma, controversy exists. The carboxy terminus of the protein was also localized at cardiomyocyte nuclei [102]. In cultured human endothelial cells, Cx43 was detected in mitochondria, where its level increased in response to cellular stress [103]. Cardiomyocyte mitochondria play an important role in triggering cardioprotection and have been suggested to act as an end-effector of the protection induced by ischemic preconditioning [104–110]. Most recently, Western blot analysis on mitochondrial preparations isolated from rat, mouse, pig (fig. 3) and human left ventricular myocardium showed the presence of Cx43 [111]. The preparations were not contaminated with markers for other cell compartments, and the localization of Cx43 to mitochondria was also confirmed by FACS sorting (double staining with MitoTracker Red and Cx43), immunoelectron and confocal microscopy. In mitochondria isolated from the ischemic anterior wall and the control posterior wall of pigs at the end of 90 min of low-flow ischemia following a preceding preconditioning cycle of 10 min of ischemia and 15 min of reperfusion, the mitochondrial Cx43/adenine nucleotide transporter ratio was more than 3-fold greater in the anterior than in the posterior wall, whereas the ratio remained at 1 in non-preconditioned myocardium [111]. The enhancement in the mitochondrial Cx43 protein level occurred rapidly, since an increase in mitochondrial Cx43 was already detected with two 5-min cycles of ischemia/reperfusion in isolated rat hearts. These data demonstrate that Cx43 is localized at cardiomyocyte mitochondria and that ischemic preconditioning enhances such mitochondrial localization. The functional importance of mitochondrial Cx43 is unknown at present.

Thus, Cx43 is involved in ischemia/reperfusion injury and the protective effect of ischemic preconditioning. The mechanism(s) underlying protection caused by Cx43, however, remain to be elucidated.

## Clinical Implications

While ischemic preconditioning is effective in healthy hearts of almost all animal species tested so far [112], there is evidence that it might be no longer operative in hearts after myocardial infarction or in failing hearts [113, 114], both entities associated with a reduction in myocardial Cx43 expression [115–118].

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## Alterations in Cardiac Connexin Expression in Cardiomyopathies

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### Abstract

Gap junctions, assembled from connexins, form the cell-to-cell pathways for propagation of the precisely orchestrated patterns of current flow that govern the synchronized rhythm of the healthy heart. As in most tissues and organs, multiple connexin types are co-expressed in the heart; the connexins Cx43, Cx40 and Cx45 are found in distinctive combinations and relative quantities in different, functionally specialized subsets of cardiomyocytes. Alterations in connexin expression and gap junction organization, now a well-documented feature of human cardiomyopathies, potentially contribute to the pro-arrhythmic substrate. In the diseased ventricle, the most consistently reported quantitative alteration involves heterogeneous reduction in Cx43 expression and disruption of the normal ordered pattern of Cx43 gap junction distribution. Additional studies suggest that upregulation of Cx40 and Cx45 may also feature in the failing ventricle, the former restricted to ischemic cardiomyopathy and localized to the sub-endocardial region. By correlating data from studies on the human patient with those from animal and cell models, alterations in connexin expression and gap junction organization have emerged as important factors to be considered in understanding the pro-arrhythmic substrate found in human cardiomyopathies.

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Cardiovascular disease is the leading cause of mortality and morbidity in most industrialized countries of the developed and developing worlds. Arrhythmias are a common, serious and often fatal complication of many forms of heart disease. As gap junctions mediate the cell-to-cell propagation of the patterns of impulse flow that govern orderly contraction of the healthy heart, increasing attention has been focused on the contribution of these junctions and their component connexins to the pro-arrhythmic substrate of the diseased heart. The literature on gap junctions and connexins in the healthy and diseased

heart is now considerable and, in addition to the coverage in this book, a range of perspectives is available in review articles [1–4]. In this chapter, we briefly review the nature and possible significance of the alterations in connexin expression and gap junctions found in human cardiomyopathies. To provide the background from which disease-related alterations can be recognized, we start with a brief reminder of the salient features of connexins and gap junctions in the normal heart.

### **Gap Junctions and Connexins in Cardiomyocytes of the Normal Heart**

Cardiac myocytes express the connexins Cx43, Cx40 and Cx45 in characteristic combinations and relative quantities according to the subtype of myocyte, location within the different chambers and developmental stage [5–10]. Overall, Cx43 predominates throughout the adult ventricular and atrial contractile myocardium though Cx40 and/or Cx45 are expressed locally at high levels, especially in the conduction system.

The working (contractile) cardiomyocytes of the ventricle are extensively interconnected by clusters of Cx43-containing gap junctions organized, together with the fasciae adherentes and desmosomal junctions, at the intercalated disks. The intercalated disks typically have a step-like configuration in which the myofibrils link to the fasciae adherentes junctions in vertical segments of the interacting disk membranes, with the gap junctions typically confined to the intervening longitudinal membrane domains. Larger gap junctions tend to populate the disk periphery. Gap junction organization together with features of tissue architecture, e.g. the size and shape of the cells, combine to facilitate preferential propagation of the impulse in the longitudinal axis and hence the normal pattern of anisotropic spread of the impulse of healthy ventricular myocardium.

Atrial cardiomyocytes are slender cells compared to their ventricular counterparts, and have shorter, less elaborate intercalated disks. The gap junctions of atrial myocytes of most mammalian species, including humans, contain abundant Cx40 [10, 11], co-localized with Cx43 within the same individual gap junctional plaques [8]. Adult working ventricular myocytes, by contrast, normally lack detectable Cx40. In both ventricular and atrial human working myocardium, Cx45 is present in low quantities, with higher levels in the atria than the ventricles [6, 10, 11].

The cardiomyocytes of the impulse generation and conduction system are quite distinct from those of the contractile ventricular and atrial cells, both morphologically [12] and with respect to their connexin expression profiles.

In experimental animals, the myocytes of the sinoatrial node, the site of impulse generation, and those of the atrioventricular node, the site at which the impulse is slowed before being directed to the ventricles, are equipped with small, dispersed gap junctions composed of Cx45 [13–15], a connexin that is known from *in vitro* experimental studies to form low conductance channels [16]. These gap junction features of nodal myocytes suggest relatively poor coupling, a property essential, for example, to the role of the atrioventricular node in slowing of conduction to ensure sequential contraction of the atria and ventricles. In the rodent, the spatial pattern of expression of Cx45 reveals that the atrioventricular node and His bundle form part of an elaborately extended central conduction system circumscribing the atrioventricular and outflow junctional regions [14].

In the rabbit sinoatrial node, a restricted zone of Cx45/Cx43 co-expression has been identified at the nodal/crista terminalis border, a putative exit route for the impulse into the atrial tissue [13]. In the atrioventricular conduction axis of this species, three-dimensional reconstructions have revealed distinctive compartmentalized connexin expression patterns. One compartment, comprising the compact node and transitional cells, expresses Cx45 while a second compartment, comprising the His bundle, lower nodal cells and posterior nodal extension, co-expresses Cx43 with Cx45 [17]. The transitional cells, which are located between atrial muscle and the compact node, connect both to lower nodal cells and the posterior nodal extension.

Downstream from the His bundle, the conduction system myocytes of most mammals, including man, prominently express Cx40, a connexin conferring high conductance when assembled into channels [6, 7, 18–20]. Prominent immunolabeling for this connexin is typical of the bundle branches of the conduction system and Purkinje fibers, correlating with the fast conduction properties that facilitate rapid distribution of the impulse to the working ventricular myocardium.

In general, different mammalian species share the key features of connexin expression described above. However, some striking exceptions have been reported, notably lack of Cx40 expression in rat atrial muscle and in the guinea pig conduction system [21, 22]. More subtle species variations are also apparent, for example, in patterns of co-expression within the atrioventricular node in larger mammals which have less need for atrioventricular nodal impulse delay than do rodents and lagomorphs [23]. Emerging data suggest that the spatial patterns of connexin co-expression along the course of the human conduction system may differ in detail from those of the mouse and rat [24]. A more detailed knowledge of species-specific patterns is important in the context of the ever wider use of transgenic animals for investigating the role of connexins in cardiac function. Extrapolation of data on transgenic mice to the human, for

example, depends critically on a sound understanding of the similarities and differences of the connexin expression of these two species [25].

### **Alterations in Connexin Expression and Gap Junctions in Cardiomyopathy**

Given that gap junctions form the cell-to-cell pathways for the precisely orchestrated patterns of current flow that govern the normal heart rhythm, a key question, first posed in the early 1990s [26, 27], was whether alterations in connexin expression and/or gap junction organization might play a role in abnormal impulse propagation and arrhythmia in human heart disease. Arrhythmias are, of course, multifactorial in origin, involving an interplay between gap junctional coupling, membrane excitability and cell and tissue architecture [28, 29]. Moreover, gap junctional coupling is itself determined by a range of factors including channel gating, the assembly/disassembly of functional gap junction plaques and the pattern, amount and types of connexin expressed. It should thus always be borne in mind that alterations to gap junctions and connexins – however critical in specific experimental instances – represent one facet of a multiplicity of interacting factors contributing to the life-threatening pro-arrhythmogenic substrate of the diseased human heart.

Experimental confirmation of a role for gap junctions in human arrhythmia in vivo is not straightforward. However, a fundamental starting point is to establish the nature of any alterations in connexin and gap junction expression associated with human cardiomyopathy and then develop and apply approaches tailored to analyze their functional significance.

In summarizing progress in these areas with respect to ventricular cardiomyopathy, it is helpful to use two (non-mutually exclusive) headings: (1) structural remodeling, which defines any alteration in the arrangement, distribution or organization of gap junctions, and (2) remodeling of connexin expression, involving alteration in the amount and/or types of connexin expressed. Two principal gap-junction-related alterations are consistently seen in the diseased ventricle: disturbances in the distribution of gap junctions, and reduced levels of their major component, Cx43. However, the detailed nature of gap junction and connexin remodeling varies according to the stage and type of cardiomyopathy.

#### *Structural Remodeling*

Alteration in the distribution of Cx43 gap junctions in the diseased human heart was first reported in the myocardial zone bordering infarct scar tissue in the ventricles of explanted hearts from patients undergoing cardiac transplantation



because of end-stage ischemic cardiomyopathy [27]. Cx43 immunolabeling in the border zone myocytes is typically scattered in disordered fashion over the lateral surfaces of the cells rather than in the regular intercalated disk arrays characteristic of normal myocardium. Electron microscopy reveals that both laterally disposed gap junctions between adjacent cells, and annular profiles of apparently internalized (non-functional) gap junctional membrane, contribute to the non-ordered Cx43 immunolabeling patterns in these human infarct border zone myocytes [27]. Gap junction disarray is not only associated with established infarct scar tissue in the human ventricle, but is initiated rapidly in response to ventricular ischemia and infarction in experimental animals [30]. Longer-term features of remodeling in myocardium distant from the infarct in the canine ventricle include reduction in the size and the number of gap junctions per unit length of intercalated disk, and fewer side-to-side connections between myocytes [31].

Lateralization of Cx43 gap junctions in a pattern resembling that in human infarct border zone myocytes has recently been reported in the ventricles of patients with compensated hypertrophy due to valvular aortic stenosis, but is not apparent in decompensated hypertrophy from the same cause; instead, a heterogeneous distribution is seen in which the gap junctions are organized largely in normal intercalated disk arrays, but with patches of tissue in which the junctions are not detectable or markedly fewer [32]. Focal disordering of Cx43 gap junctions is also found in small areas of the explanted ventricle in transplant patients with heart failure due to idiopathic dilated cardiomyopathy and myocarditis [33]. Lateralization features in some models of ventricular hypertrophy in the rat [34, 35] and, in one model, is associated with reduced longitudinal conduction velocity [35]. Quite spectacularly disordered arrangements of ventricular Cx43 gap junctions are found between the haphazardly organized myocytes characteristic of human hypertrophic cardiomyopathy, the most common cause of sudden cardiac death due to arrhythmia in young adults [36].

A yet different form of structural remodeling is associated with human hibernating myocardium in patients with ischemic heart disease [37]. The term 'hibernating myocardium' defines a region of ventricular myocardium that shows impaired contraction but which has the capacity to recover contractile function after coronary artery bypass operation. In human hibernating myocardium, the large Cx43 gap junctions typically found at the periphery of the intercalated disk are smaller in size, and the overall amount of Cx43 immunolabeling per intercalated disk is reduced, compared with normally perfused (and reversibly ischemic) segments of the same heart [37]. These observations led to the hypothesis that Cx43 gap junction remodeling is linked to impaired ventricular contraction, as well as arrhythmia, in the diseased human ventricle [37].

### *Alterations in Connexin43 Expression*

As emphasized by the above observations on hibernating myocardium, disease-related structural remodeling is often accompanied by alterations in the amount of connexin expressed. The most thoroughly and widely documented disease-related alteration in ventricular connexin expression involves downregulation of Cx43; a significant reduction in Cx43 transcript and protein levels is apparent in the left ventricles of transplant patients with end-stage congestive heart failure regardless of whether the condition is due to idiopathic dilated cardiomyopathy, ischemic cardiomyopathy, valvular aortic stenosis or other etiologies [32, 33, 38–40]. The reduction in ventricular Cx43 appears to develop progressively during the course of disease, as suggested by the pattern of change observed in pressure-overloaded hearts with valvular aortic stenosis classified according to ejection fraction [32] and the finding of reduced Cx43 in the non-failing ventricles of patients with ischemic heart disease undergoing coronary artery bypass surgery [41]. Recent evidence [32] raises the possibility that the long-term disease-related decline in Cx43 may be preceded by an adaptive increase in the connexin during the early stage of compensated hypertrophy.

The functional significance of reduced ventricular Cx43 levels in cardiomyopathy has been the subject of lively debate. In any such discussion, it should be borne in mind that while total connexin levels may reflect the *potential* capacity for cell-to-cell communication or coupling, they give no indication of the quantity of functional (open) channels. This, combined with predictions from computer modeling that reductions of up to 40% in gap junction content (without change in junction size) would have no significant effect on conduction velocity [4], might suggest that a reduction in this magnitude of Cx43 alone would in theory confer no adverse functional consequences on the diseased ventricle. However, reduction in connexin levels is neither a sole nor uniform change, even within the context of gap junction remodeling. In view of the complex relationship between passive and active membrane properties, the multiplicity of structural and functional alterations in the diseased heart and the assumptions inherent in computer modeling, in vivo extrapolation of the effects of a single change (i.e. reduced Cx43 levels), in isolation from other factors, may not give the full picture.

Studies on experimental animals shed further light on this issue. In a transgenic mouse model of juvenile dilated cardiomyopathy, reduced Cx43 and conduction defects reportedly become apparent 4 weeks after birth, with contractile dysfunction and heart failure following at 12 weeks [42]. In intact isolated hearts of transgenic mice expressing half the normal level of Cx43, experimental ischemia reportedly leads to a marked increase in incidence, frequency and duration of ventricular tachycardias [43] even though reduction in conduction velocity is modest [44]. On the other hand, in transgenic mice generated to give

cardiac-specific loss of Cx43, sudden death due to spontaneous ventricular arrhythmia does not occur until an 86–95% reduction in Cx43 is reached [45], much lower than the average reduction found in the diseased human ventricle (~50%). An important feature to emphasize is that Cx43 reduction in the failing human ventricle is not uniform; a considerable variation in the extent of Cx43 reduction is found between and, in particular, within hearts, some regions of some diseased hearts reaching a reduction of >90% of control values [38] (i.e. similar to the levels at which fatal cardiac arrhythmia occurs in the cardiac-restricted Cx43 knockout mouse). Thus, average values for the overall reduction in ventricular Cx43 in the diseased human heart disguise considerable spatial heterogeneity in the extent of the reduction. It is the existence of this heterogeneity, arising from a combination of altered gap junction arrangement and reduction in Cx43, that is now considered critical. Heterogeneous reduction in Cx43 could quite plausibly lead to exaggeration of inhomogeneities in resting potential and action potential upstroke velocity and duration, affecting individual cell excitability and refractory period, dispersion of which is a key pro-arrhythmic factor. Inhomogeneous wave front propagation could in turn lead to asynchronous myocyte contraction and poor ventricular force development.

Experimental studies have demonstrated the importance of heterogeneity of cardiac Cx43 expression in relation to disturbances in electromechanical function [46]. In chimeric mice, created from Cx43-deficient stem cells and blastocysts, a patchy expression of ventricular Cx43 is obtained which mimics some of the remodeling patterns found in the diseased human ventricle. These experimental mice were demonstrated to have abnormal conduction and marked contractile dysfunction [46], just as originally hypothesized in the human studies [27, 37, 38]. A further refinement has been the development of outbred cardiac-restricted knockouts, which show a more gradual decline in Cx43 levels than the original cardiac-restricted knockout animals [47]. These experimental animals have permitted closer definition of the precise level at which Cx43 reduction becomes critical. By 45 days after birth, Cx43 levels show a heterogeneous reduction to 18% of control levels, conduction velocity is slowed to half that of control animals, and 80% of the experimental animals are inducible into lethal ventricular arrhythmias [47].

Taking all these findings together, there is now a considerable body of evidence to suggest that spatially heterogeneous reduction in Cx43 of the magnitude and nature observed in the diseased human ventricle may indeed contribute to the pro-arrhythmic substrate. Precisely how reduction in Cx43 expression and gap junction size and number are brought about in the setting of human cardiac disease, however, remains a major unanswered question; among candidate mechanisms are those involving the PDZ-MAGUK protein ZO-1, growth factors such as vascular endothelial growth factor, and activation of the

stress-activated protein kinase, c-Jun N-terminal kinase (JNK) [for a review, see ref. 1]. JNK activation in a transgenic mouse model leads to an impressive combination of loss of Cx43, slowing of ventricular conduction, contractile dysfunction and congestive heart failure [48].

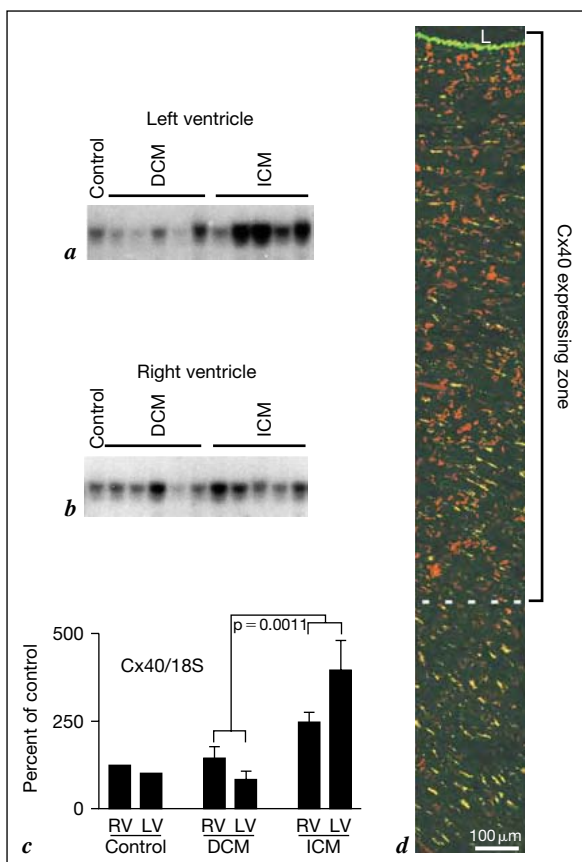
#### *Alterations in the Expression of Connexins45 and 40*

Fewer studies have documented alterations in the expression of connexins other than Cx43 in the diseased human ventricle, though elevated levels of Cx45, and in some instances also Cx40, are reported to feature. In parallel with the reduction in Cx43 in the failing human ventricle, Yamada et al. [40] report an upregulation of Cx45. As, in human ventricle, Cx43 and Cx45 commonly appear co-localized in the same gap junctional plaque [10, 40], the possibility exists that these two connexins may be assembled into heteromeric connexons and channels. Cx43/Cx45 heteromeric channels are reported to have reduced unitary conductance compared with that of homomeric Cx43 channels [49]. Thus, even though the overall levels of Cx45 are low, elevated Cx45 in the failing ventricle might, it was argued, exert a significant effect on channel properties through altered Cx43/Cx45 stoichiometry, and thereby possibly creating microheterogeneities in coupling [40].

Increased levels of Cx40 transcript have been detected in the ventricles of patients with congestive heart failure due to ischemic cardiomyopathy but not that due to idiopathic dilated cardiomyopathy [38]. This elevated Cx40 expression correlates with an increased depth of Cx40-expressing myocytes from the endocardial surface, in the vicinity of the Purkinje myocytes (fig. 1). Whether this change represents an expansion of the Purkinje system or a transformation of working myocytes to become more Purkinje like is unclear. The significance of this expanded zone of Cx40 expression, which shows some similarity to that reported in ventricular hypertrophy in the rat [18], is unknown. One possibility is that it might represent some form of adaptive response in the face of declining Cx43 levels.

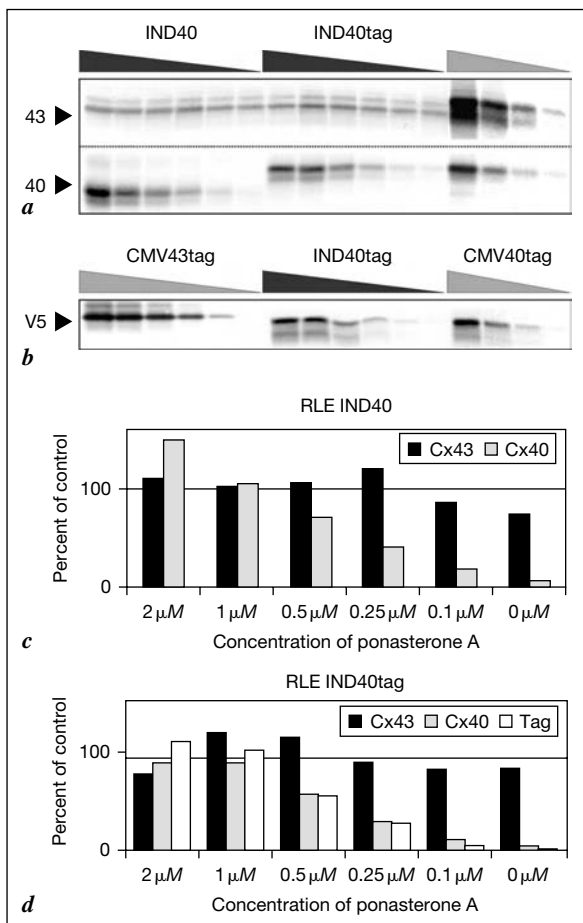
#### **Analyzing the Significance of Altered Connexin Co-Expression**

Though plausible ideas may be advanced for the functional significance of increased levels of Cx40 or Cx45 alongside reduced Cx43, these can remain no more than speculations unless subjected to direct experimental test. As indicated earlier in this chapter, the intact human heart is not readily amenable to such experimental investigation. We have therefore developed an in vitro model, designed to express the same combinations of connexins in the same

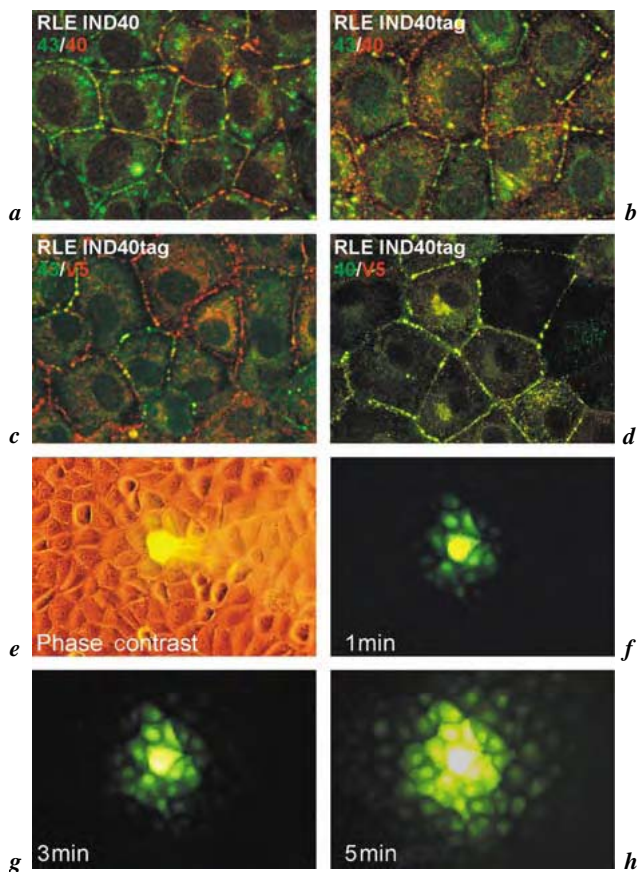


**Fig. 1.** *a, b* Northern blot analyses demonstrating upregulation of Cx40 transcripts in the left (*a*) and right ventricle (*b*) of patients with ischaemic heart disease (IHD) but not those with idiopathic dilated cardiomyopathy (DCM). *c* Quantification of northern blot data. *d* Immunofluorescence microscopy demonstrates increased depth of expression of Cx40 protein (red fluorescence) from the endocardial surface in the IHD patients. L = Ventricular lumen.

relative quantities as those found in defined cardiac tissues and disease states in vivo, but in which parallel functional studies can be conducted. These models are based on the use of an inducible plasmid system that enables the co-expression of connexins under the control of ecdysone- and tetracycline-inducible promoters [50]. An internal ribosome entry site sequence linking the connexin to an antibiotic resistance gene (i.e. producing a bicistronic mRNA transcript) is used to ensure homogeneity of expression in the cells [50]. RLE (rat liver epithelial) cells are particularly suitable for use in our model as they give a uniform



**Fig. 2.** *a, b* Western blot analyses of connexin expression response under the control of the ecdysone system. The immunoblot shows analysis of endogenous Cx43 in the transfected Cx40 (Ind40) and Cx40-tag (Ind40tag) cell lines cultured at different levels of induction (black gradient indicates concentration of ponasterone A from 2 to 0  $\mu\text{M}$ , *a*). The second set of blots shows Cx40 in the same samples. Note progressive reduction in the amount of Cx40 expressed as the ponasterone A concentration is decreased. The set of blots under 'Cmvtag' show data from which a standard curve is produced (as indicated by the grey gradient). The addition of the V5 tag to the Cx40 (*b*) gives a common standard throughout, allowing the relative abundance of different connexins to be quantified in relation to each other. *c, d* The bar charts show quantification of expression of Cx43 and Cx40 relative to each other. Analyses of Cx40 transfectants (*c*) and cells transfected with Cx40-tag (*d*) are shown. The immunoreaction was normalized to the mean endogenous expression of Cx43 of the samples (shown as 100%).



**Fig. 3.** *a–d* Immunofluorescence images of RLE cells transfected to express Cx40 or its V5 tagged counterpart alongside endogenous Cx43. *a* Double labelling with anti-Cx43 (green) and anti-Cx40 (red) confirms expression of both connexins in a pattern that varies from one interface to the next. Within a given cell, some interfaces show predominantly Cx43 label, while others show predominantly Cx40 or a mixture of Cx40 and Cx43. *b, c* This pattern is reproducible when Cx40 is replaced by V5-tagged Cx40 and when an anti-tag antibody is used in place of the Cx40 antibody. *d* Simultaneous application of both the anti-Cx40 antibody and the V5 antibody results in yellow label, i.e. the expressed Cx40-tag is equally labeled by both antibodies, thus confirming the fidelity of the expression patterns in *a–c*. The colored symbols on the figures represent the color of the fluorochrome used to detect the indicated target (i.e. Cx43, Cx40 or V5 tag). *e–h* Microinjection of Lucifer yellow in RLE Cx40 transfectants at high level of induction show rapid cell-to-cell spread of the tracer, indicating high levels of gap junctional communication. Time indicates period after introduction of micropipette.

monolayer, endogenously express high levels of Cx43 and have a high level of intercellular communication.

By transfecting the cells with the chosen connexin under the control of the ecdysone promoter, fully inducible Cx40 clones and Cx45 clones have been obtained. The expression level of the transfected connexin is readily controlled at will (fig. 2), and the cells show overall uniform expression of the endogenous and transfected connexins. Accurate determination of the ratio of the co-expressed connexins is achieved by introducing a V5 6xhistidine tag at the connexin C terminus which can be used to calibrate relative expression by Western blot. At the higher levels of induction, it should be noted that expression of the transfected connexin can be manipulated to levels both above and below that of endogenous Cx43. Thus, it is possible to mimic the patterns of increased Cx40:Cx43 and of increased Cx45:Cx43, as reported in human cardiomyopathy.

Close inspection of the transfectants after double labeling for Cx43 and Cx40 reveals that within an overall homogeneous expression of the two connexins, distinct patterns of expression can occur at different cell interfaces, even within a single cell (fig. 3a–d). This raises the possibility that the Cx40:Cx43 ratio within a connexon influences compatibility of heteromeric connexon docking. A number of functional analyses can be applied to these cells. Microinjection of small tracer molecules (fluorescent dyes such as Lucifer yellow and ethidium bromide) permits comparison of the permeability properties of the gap junctional channels in cells with increased Cx40:Cx43 and increased Cx45:Cx43 (fig. 3e–h). The next step will be to determine intracellular resistance, a major determinant of conduction velocity in myocardium. All functional data will then be related to structural data on the stoichiometry of the heteromeric connexons/channels made in co-expressing cells. A further refinement will be to extend these studies to HL-1 cells (a cardiac muscle cell line) [51] to allow the direct measurement of conduction velocities in relation to increased Cx40:Cx43 and increased Cx45:Cx43 ratios.

### **Concluding Comment**

To conclude, remodeling of myocyte connexins and gap junctions – notably heterogeneous reduction in ventricular Cx43 and disordering in the pattern of junctional distribution – feature prominently in cardiomyopathy, and similar alterations correlate with electrophysiologically identified pro-arrhythmic changes and contractile dysfunction in animal models. Increased expression of Cx45 and Cx40 may, at least in some instances, occur in parallel with the reduction in Cx43. The major task, currently in hand, is to define the functional



consequences of these alterations in connexin expression in the diseased human heart.

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## Alterations of Connexin 43 in the Diabetic Rat Heart

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### Abstract

In the streptozotocin-induced diabetic rat heart, a decrease in the conductivity and suppression of electrical cell-to-cell coupling has been observed. To clarify this mechanism, the present study was performed to investigate the gap junction connexin 43 (Cx43) using immunohistochemistry, immunoblot, electron-microscopic analyses. Enhanced activation of PKC $\epsilon$ , augmentation of PKC $\epsilon$ -mediated phosphorylation of Cx43, a decrease in the total amount of Cx43, a reduction in the number of immunoreactive particles for Cx43 at the intercalated disk and internalization, annular profiles of the gap junction were all recognized in the diabetic heart. Such a deterioration in the expression of Cx43 was alleviated by treatment with either lysosomal (leupeptin) or proteasomal inhibitor (ALLN). These results suggest that the PKC $\epsilon$ -mediated hyperphosphorylation of Cx43 makes Cx43 vulnerable to proteolytic degradation, while a decrease in the conductivity in the diabetic heart is also caused by a decrease in the number of gap junction channels due to an acceleration of the proteolytic degradation of Cx43. The remodeling of Cx43 induced by the activation of PKC $\epsilon$  may therefore contribute to the formation of the arrhythmogenic substrate.

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In cardiac muscle, the gap junctions greatly contribute to electrical cell-to-cell coupling. A dysfunction in the gap junction impairs conductivity and can thus be one of the arrhythmogenic factors inducing re-entrant excitation. The physiological function of the gap junctions essentially depends on the characteristics of the channels which comprise the gap junction, as well as the number of channels or channel open probability. These factors depend on the phosphorylation of the connexins, since connexins are phosphoproteins.

Abnormalities in the intraventricular conduction and susceptibility to arrhythmias have been observed in diabetic patients [1–3].

Furthermore, PKC is activated in the diabetic heart [4–8], in diabetic vascular tissues [9–11] and in vascular cells exposed to a high glucose medium [12]. These observations suggest that a relationship exists between the activation of PKC and the intercellular conduction disturbances caused by alterations in the gap junction function, since the activation of PKC impairs the intercellular communication through the gap junction [13–23]. The dysfunction of the gap junction induced by remodeling of the connexin [24, 25] might thus explain the higher arrhythmogenicity observed in the diabetic heart [24, 25]. On the other hand, the precise isoform of PKC that is activated in the diabetic heart remains controversial and remains to be elucidated.

Therefore, in this study, the identification of the PKC isoform and alterations in the phosphorylation of connexin were examined in the streptozotocin (STZ)-induced diabetic rat heart, by means of electrophysiology, immunoblotting and immunohistochemistry, with reference to the gap junction function. The experiments focused on connexin 43 (Cx43) which is a major gap junction protein in ventricular cells.

Part of this study was previously reported in abstract form [26].

## Methods

### *Materials and Preparation of the Samples*

A diabetic state was induced by STZ (50 mg/kg, as a single i.v. injection, after 24 h of fasting) in adult male Wistar rats weighing  $225.5 \pm 13.2$  g, and after 3 or 4 weeks in a diabetic state (blood glucose over 400 mg/dl) the rats were sacrificed. Age-matched animals were used as a normal controls.

At 30 min after heparinization, the animals were sacrificed by a blow to the head, and this methodology was approved by the Institutional Animal Care and Use Committees of Fukuoka University. Next, after opening the thorax, the heart was quickly removed and mounted on a Langendorff apparatus which was well perfused with oxygenated (97% O<sub>2</sub>, 3% CO<sub>2</sub>) Krebs-Henseleit solution at constant pressure (60 mm Hg) and constant flow (20 ml/min). After steady state of force for ventricular contraction and sinus regular rhythm was obtained (usually within 5 min), the experiments were started.

Tissue samples extracted from both right and left ventricular tissues were frozen in liquid nitrogen for Western blotting or were immersed in the fixative (3% buffered paraformaldehyde) for immunohistochemical and (3% buffered glutaraldehyde) for electron microscopic examinations. For the in vitro experiments, a thin muscle strip was isolated from the right ventricular endocardium.

### *Evaluation of Electrical Cell-to-Cell Coupling*

To evaluate the functional cell-to-cell coupling, the conduction velocity and the longitudinal internal resistance were measured in the isolated muscle preparation as previously described [27].

### *Immunoblot and Immunohistochemistry of Cx43*

In Western blotting, specific mouse monoclonal anti-Cx43 antibody (Chemicon Int. Inc., USA) (for the detection of the phosphorylated and unphosphorylated isoform) and mouse monoclonal

anti-Cx43 antibody (Zymed Lab. Inc. USA) (for the detection of un-phosphorylated isoform) were used at dilutions of 1:4,000. Secondary antibody (Donkey anti-rabbit IgG, Chemicon Int. Inc., USA) was used at dilutions of 1:5,000. The Western blotting procedures have been previously described [27].

The total amount of Cx43 was evaluated by means of the density of the un-phosphorylated isoform detected by either the monoclonal antibody or by alkaline phosphatase treatment using the monoclonal antibody.

In immunohistochemistry, rabbit polyclonal anti-Cx43 antibody (Zymed Lab. Inc., USA) was used at dilutions of 1:200 and goat anti-rabbit IgG (Alexa Fluor 488, Molecular Probe, USA) was used at dilutions of 1:200 as a secondary antibody. Immunofluorescence was detected using a confocal laser scan microscope (LSM-410, Carl Zeiss).

In the electron microscopic examination, conventional methods were used as previously described [28].

The mean density of the Cx43 complex isoforms in the immunoblots and the mean fluorescent intensity of immunoreactive particles for Cx43 were analyzed by NIH Image Software.

#### *Chemical Reagents*

Phorbol-12-myristate 13-acetate (PMA) (Sigma) ( $0.1 \mu M$ ) was used as an activator of PKC, Calphostin C (RBI) ( $1 \mu M$ ) as PKC inhibitor, as lysosomal inhibitor, leupeptin (acetyl-leu-leu-arg-al) (Sigma) ( $25 \mu M$ ), and as proteasomal inhibitor, N-acetyl-leu-leu-norleucinal (ALLN) (Sigma) ( $25 \mu M$ ). These reagents were dissolved in DMSO immediately before use. They were used in perfused heart on Langendorff for 60–90 min at the final concentrations described above. The identification of PKC isoforms was performed by Western blotting using polyclonal antibody for PKC $\alpha$  (C-20, sc-208),  $\beta 1$ (C-16, sc-209),  $\beta 2$ (C-18, sc-210),  $\delta$ (C-17, sc-213),  $\epsilon$ (C-15, sc-214),  $\theta$ (C-18, sc-212) (Santa Cruz Biotechnology, Inc.). Alkaline phosphatase (from bovine intestinal mucosa, Sigma) was added in the first homogenizing procedure at a dose of 20 U/ml.

#### *Statistical Analysis*

The data were expressed as the mean  $\pm$  SEM. The un-paired Student's t test was used to analyze any statistical significance ( $p < 0.001$ ) between the means.

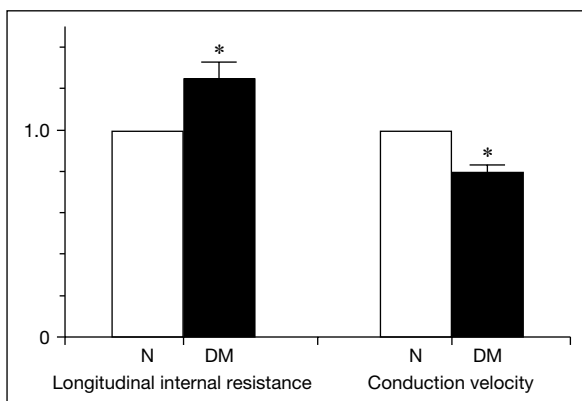
## **Results**

### *Electrical Cell-to-Cell Coupling*

Figure 1 shows a comparison of the conduction velocity and the longitudinal internal resistance of the right ventricular endocardium between the normal and the diabetic heart. The conduction velocity and the longitudinal internal resistance significantly decreased and increased, respectively, in the diabetic heart in comparison to the normal heart.

### *Expression of Cx43 at the Intercalated Disk*

As shown in figure 2a, Cx43 immunoreactive particles were sparse and only sporadically observed at the intercalated disk and they were also seen on the surface membrane in the diabetic heart. The results of statistical analyses of the immunoreactive intensity and area of the immunoreactive particles at the intercalated disk are shown in figure 2b. Both the intensity and the area significantly decreased in the diabetic heart.



**Fig. 1.** Comparison of the longitudinal internal resistance and the conduction velocity between normal (N) and diabetic (DM) hearts. The values are relative values (1.0 represents the value for normal hearts). Vertical bars represent the mean  $\pm$  SEM and \* $p < 0.001$  vs. normal hearts.

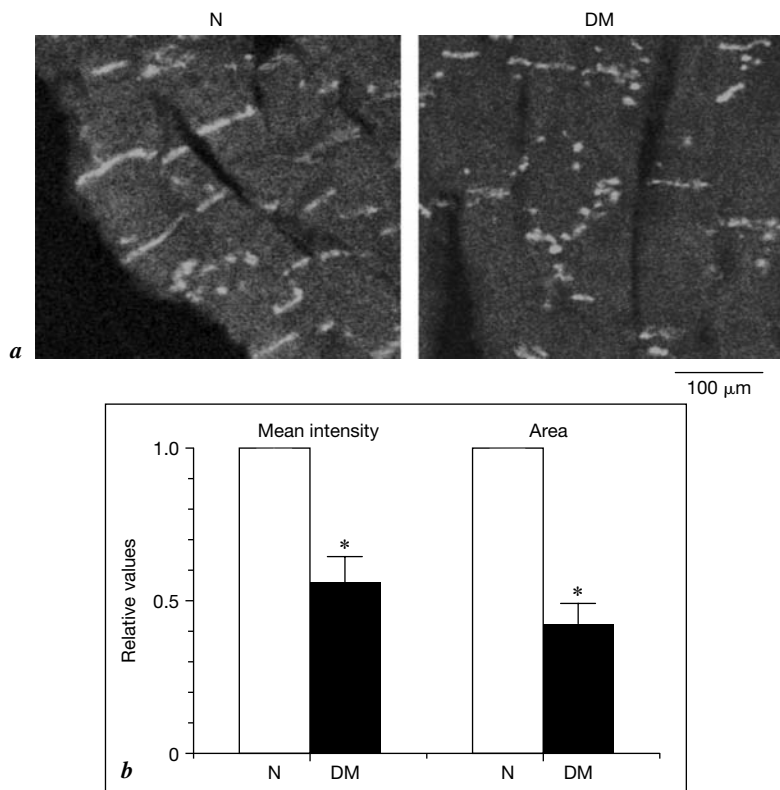
### *Phosphorylation of Cx43*

In the Western blottings for Cx43, three isoforms were visible in both the normal and the diabetic heart (fig. 3a). Two higher molecular isoforms were abolished after treatment with alkaline phosphatase; however, one lower molecular isoform was not (not shown here). Therefore, the higher molecular isoforms were classified as phosphorylated isoforms of Cx43, P1, P2, and the lower molecular isoform, as an un-phosphorylated isoform, P0.

In the diabetic heart, P2 was remarkably augmented. The expression of P2 decreased following Calphostin C treatment. In other experiments, an augmentation of P2 was also observed at 90 min after the application of PMA (0.1  $\mu$ M) and the P2 band was also abolished by pretreatment with Calphostin C (not shown here). As a result, the P2 isoform of Cx43 is regarded as PKC-mediated phosphorylated Cx43. The P2/P0 ratio evaluates the degree of PKC-mediated phosphorylation of Cx43. The results of a densitometric analysis of the P2/P0 showed an augmentation of the PKC-mediated phosphorylation of Cx43 in the diabetic heart (fig. 3b). The total amount of Cx43 decreased in the diabetic heart in comparison to that in the normal heart (fig. 3c, d)

### *Identification of the PKC Isoform That Is Activated in the Diabetic Heart*

The isoforms of PKC $\alpha$ , PKC $\beta_1$ , PKC $\beta_2$ , PKC $\delta$ , PKC $\epsilon$  and PKC $\theta$  were detected by Western blotting in the diabetic heart more frequently than in



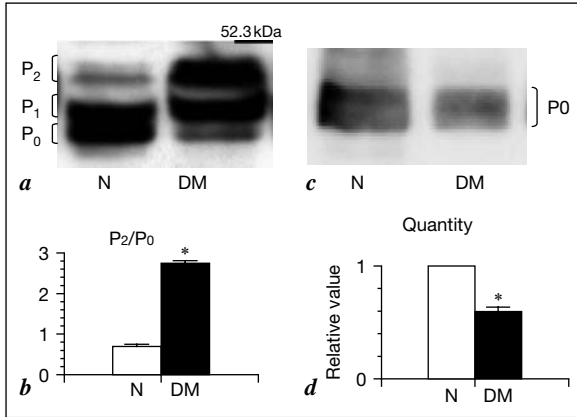
**Fig. 2.** *a* Confocal laser scan micrographs of the immunofluorescence of Cx43. N = Normal; DM = diabetic. *b* Analysis of the immunofluorescence of Cx43. Comparison of the mean intensity and area of the immunoreactive particles at the intercalated disk between normal (N) and diabetic (DM) hearts. The columns represent relative values (1.0 represents the value for normal hearts). Vertical bars represent the mean  $\pm$  SEM and \* $p < 0.001$  vs. normal hearts.

the normal heart (fig. 4a). The results of a densitometric analysis are shown in figure 4b. Only the PKC $\epsilon$  isoform was augmented in the diabetic heart, namely, PKC $\epsilon$  was activated in the diabetic heart. The co-immunoprecipitation of Cx43 and PKC $\epsilon$  revealed that the PKC-mediated hyperphosphorylation of Cx43 in the diabetic heart was mediated by PKC $\epsilon$  (fig. 4c).

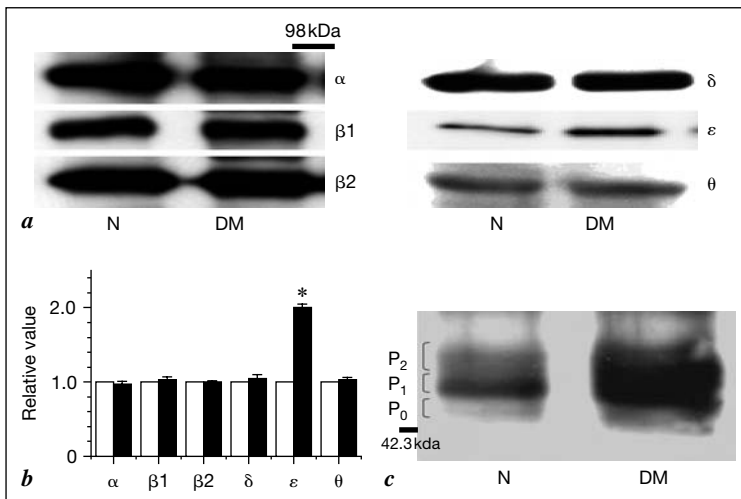
#### *Effects of Lysosomal and Proteasomal Inhibitors on the Expression of Cx43 in the Diabetic Heart*

The effects of leupeptin on the immunoreactive expression of Cx43 are shown in figure 5a. The deteriorated expression of immunoreactive particles for

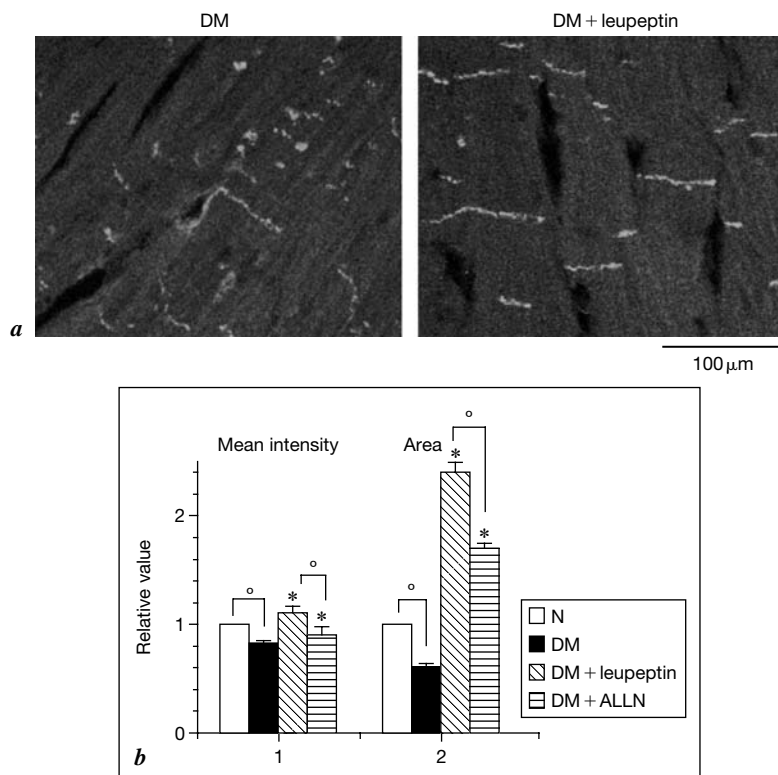




**Fig. 3.** *a* Western blots of Cx43. N = Normal; DM = diabetic. *b* Densitometric analysis of the immunoblot. Comparison of the P2/P0 ratio between normal (N) and diabetic (DM) hearts. *c* Western blots of Cx43 after treatment of alkaline phosphatase. *d* Comparison of the quantity of Cx43 protein between normal (N) and diabetic (DM) hearts according to densitometric analysis of *c*. The columns represent the relative values (1.0 means value of the normal hearts). Vertical bars represent the mean  $\pm$  SEM and \* $p < 0.001$  vs. normal hearts.

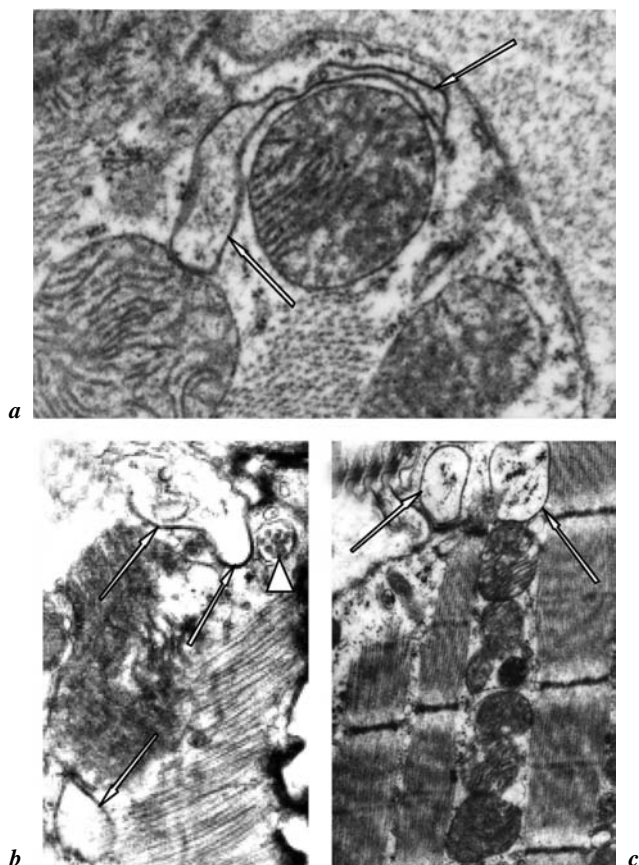


**Fig. 4.** *a* Western blots of PKC isoforms in normal (N) and diabetic (DM) hearts. *b* Densitometric analysis of the immunoblots of PKC isoforms. The columns represent relative values (1.0 represents the value for normal hearts). Vertical bars represent the mean  $\pm$  SEM and \* $p < 0.001$  vs. normal hearts. *c* Co-immunoprecipitation of Cx43 and PKC $\epsilon$  in normal (N) and diabetic (DM) hearts.



**Fig. 5.** *a* Confocal laser scan micrographs of the immunofluorescence of Cx43. DM = Diabetic hearts; DM+leupeptin = control diabetic hearts treated with leupeptin. *b* Analysis of the immunofluorescence findings of Cx43. Comparison of the mean intensity and area of the immunoreactive particles at the intercalated disk between the normal (white column), control diabetic (black), diabetic heart treated with Leupeptin (shadow) and ALLN (dotted). The columns represent the relative values (1.0 mean value of the normal hearts). Vertical bars represent the mean  $\pm$  SEM and \* $p < 0.001$  vs. the normal hearts. ° $p < 0.001$ .

Cx43 at the intercalated disk in the diabetic heart was alleviated by treatment with leupeptin. A proteasomal inhibitor, ALLN, demonstrated the same ameliorative effects as those of leupeptin, but its effect was less potent than that of leupeptin (immunofluorescence micrographs, not shown here). A statistical analysis of the mean intensity and the area of the immunoreactive particles of Cx43 are shown in figure 5b. The application of lysosomal inhibitor as well as proteasomal inhibitor enabled the deteriorated expression of Cx43 to substantially recover at the intercalated disk.



**Fig. 6.** Electron micrographs of a sample from a diabetic heart. The internalization and annular profiles of the gap junctions are represented (*a-c*, arrows). Note the location of the gap junction in the vicinity of the mitochondria (*a-c*) as well as the multivesicular bodies (arrow head in *b*).

### *Morphological Alterations of the Gap Junction in the Diabetic Heart*

Electron micrographs of the diabetic heart are shown in figure 6a-c. The internalization and annular profiles of the gap junctions localized in the vicinity of mitochondria were characteristically observed in the diabetic heart.

## Discussion

Previous clinical reports have shown diabetic patients to be susceptible to ventricular arrhythmias due to conduction disturbances, such as a conduction delay or conduction block [1–3].

The conduction velocity has been well documented to depend on the maximum  $dV/dt$  of the action potential. In the diabetic heart, the maximum  $dV/dt$  did not significantly decrease in comparison to the normal heart. The conduction velocity and the longitudinal internal resistance can be evaluated as electrical cell-to-cell coupling, thereby reflecting the electrical resistance of the gap junction [29]. Evidence that a reduction in the conduction velocity and an increase in the longitudinal internal resistance in the diabetic heart suggest that a deterioration in the conductivity of the diabetic heart is possibly caused by an impairment of the gap junction function, since the gap junction plays a main role in the intercellular impulse propagation.

Immunohistochemistry for Cx43 revealed that the number of Cx43 immunoreactive particles at the intercalated disk decreased and the immunopositive spots were sparse and sporadic in the diabetic heart. These abnormalities of the Cx43 expression in the gap junction in the diabetic heart have been observed in previous reports [28]. These findings suggest that the number of the Cx43 gap junction channels decreased in the diabetic heart. Such a reduction in the number of gap junction channels supports the existence of an impairment in the conductivity of the diabetic heart. These events also coincide with a reduction in the total amount of Cx43.

A Western blot analysis for Cx43 indicated the PKC-mediated phosphorylation of Cx43 to be augmented in the short-term diabetic heart. PKC-mediated hyperphosphorylation of Cx43 was also documented in diabetic vascular tissues [11], phorbol-ester-treated vascular tissues [30] or vascular tissues immersed in high-glucose medium [12, 30] and in other phorbol-ester treated tissues [22, 31, 32].

The identification of the isoform of PKC that is activated in diabetes remains controversial. PKC $\alpha$  [8], PKC $\beta$  [11], PKC $\beta_2$  [7], PKC $\delta$  [8] or PKC $\epsilon$  [6] is activated in the heart, while PKC $\beta$  is activated in vascular tissues [9, 10]. In this study, PKC $\epsilon$  was confirmed to be activated in the diabetic rat heart. The results of the co-immunoprecipitation of Cx43 and PKC $\epsilon$  indicate that the hyperphosphorylation of Cx43 is mediated by PKC $\epsilon$ .

It is interesting to note that a reduction in the total quantity of Cx43 protein and a decrease in the number of immunoreactive particles are both associated with the PKC-mediated hyperphosphorylation of Cx43. It is also probable that when Cx43 is excessively phosphorylated by PKC it is susceptible to proteolytic degradation, as described in previous reports [31, 33, 34]. This possibility is supported by the present results which show the reduced quantity of Cx43

and deteriorated expression of Cx43 in the diabetic or PMA-treated heart are improved following treatment with lysosomal or proteasomal inhibitors, since gap junction connexin is degraded by either the lysosomal pathway, the proteasomal pathway or both [35–40]. On the other hand, the internalization or annular profile of the gap junction also represents processes of proteolytic degradation via the lysosome pathway, the proteasome-dependent pathway or both [22, 40–42]. In the diabetic heart, such ultrastructure alterations, internalization and annular profiles were recognized in this study. These results suggest that in the diabetic heart the proteolytic degradation of Cx43 is accelerated by the hyperphosphorylation of Cx43 due to the activation of PKC $\epsilon$ . In tissue specimens other than the heart, the hyperphosphorylation of Cx43 due to PKC activation was found to induce either a degradation of Cx43 [31, 34] or a loss in the gap junction communication [11, 22].

The activation of PKC induces a reduction in the intercellular communication through the gap junction (a decrease in gap junction conductance, gj; a suppression of dye-coupling) due to the phosphorylation of connexin [13–19, 22, 23]. However, the mechanism for this action has yet to be clarified. The closing of the channels [15], a decrease in the channel open probability [15], a reduction in the number of channels (inhibition of the synthesis of connexin-connexon, impairment of the assembly of connexon into the gap junction plaque or acceleration of proteolytic degradation) are all involved in this mechanism [43]. Our findings demonstrated the possible acceleration in the degradation in the Cx43.

In conclusion, an impairment in the conductivity in the diabetic heart is possibly caused by loss in the gap junction channels due to the acceleration of proteolytic degradation of Cx43 resulting from the activation of PKC $\epsilon$ . The remodeling of Cx43 induced by the activation of PKC $\epsilon$  thus becomes the arrhythmogenic substrate, thereby resulting in the heart being susceptible to cardiac arrhythmias.

## Acknowledgements

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## Connexins in Atherosclerosis

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### Abstract

Remodeling of the vascular wall plays a central role in many physiological processes, but also in the pathogenesis of cardiovascular diseases such as atherosclerosis and restenosis. Atherosclerosis represents the major cause of death and disability in adult populations of Western societies. Angioplasty is a common and effective method of treatment for coronary atherosclerosis, but restenosis after the procedure continues to be a serious clinical complication. The development of atherosclerosis and restenosis involves complex patterns of interactions between the dysfunctional endothelium, inflammatory cells and smooth muscle cells in which cytokines and growth factors are known to play a critical role. Apart from paracrine cell-to-cell signaling, a role for gap-junction-mediated intercellular communication has recently been suggested. In this chapter, we summarize existing evidence supporting such a role. Thus, the pattern of vascular connexins is altered during atherosclerotic plaque formation and in restenosis. In addition, disturbances in flow, inflammation and smooth muscle cell activation and proliferation have been shown to affect connexin expression or gap junctional communication. Finally, genetically modified connexin expression alters the course of these diseases in mice. Further studies will tell us whether future treatment of atherosclerosis or restenosis may involve connexin-based strategies.

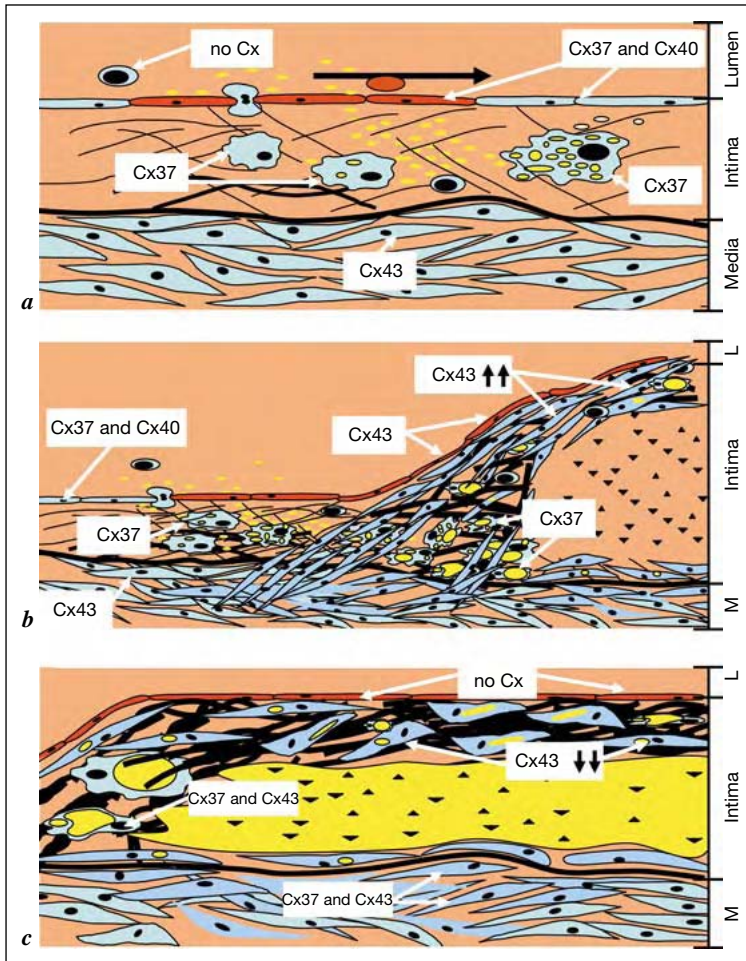
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### Current Concepts of Atherosclerosis and Restenosis

Atherosclerosis is defined as a chronic immuno-inflammatory disease characterized in part by the accumulation of lipids, leukocytes, and smooth muscle cells (SMCs) in the intima of large and medium-sized arteries [1–3]. This multifactorial and progressive disease is the leading cause of illness and death in developed countries. Atherosclerosis is promoted by a variety of risk factors including hypercholesterolemia, obesity, smoking, hypertension, diabetes and infections. These factors are linked by their common ability to promote





**Fig. 1.** Connexin expression during atherosclerotic plaque development. ***a*** Cx37 and Cx40 are expressed in healthy ECs (blue), Cx43 in medial SMCs; connexins are not detected in circulating monocytes. As a response to injury, for example by LDL (yellow dots) and turbulent flow (black arrow), the endothelium becomes dysfunctional (red) but still expresses Cx37 and Cx40. Cx37 expression is induced in monocytes/macrophages as soon as they have passed the endothelial barrier. ***b*** Chemokines secreted by inflammatory cells in the vascular wall induce the recruitment of more monocytes. This inflammatory reaction induces proliferation and migration of the SMCs from the media to the intima, finally forming a fibrous cap covering the atherosclerotic lesion. Activated SMCs display increased levels of Cx43 expression. The ECs in the shoulder region of the atheroma no longer express Cx37 and Cx40 but Cx43 instead. Macrophages take up oxidized LDL, become macrophage foam cells and still express Cx37. ***c*** A lipid core is formed by necrotic debris, extracellular lipids and cholesterol crystals. Foam cells close to the lipid core express Cx37 and Cx43. SMCs in the

inflammatory reactions and injury to the endothelium. As a response to injury, the endothelium becomes dysfunctional, leading to increased expression of various cell adhesion molecules and secretion of chemoattractants to recruit specific leukocytes. Leukocyte recruitment in the early phases of atherosclerosis mainly involves monocytes; however, T lymphocytes are also implicated in the development of the disease. After adhering to the dysfunctional endothelium, monocytes transmigrate across intact endothelial cells (ECs) to penetrate into the arterial intima, as illustrated in figure 1a. In the intima, monocytes proliferate and mature under the influence of cytokines, chemokines and growth factors secreted by themselves and other atheroma-associated cells. Furthermore, the induced expression of scavenger receptors permits macrophages to accumulate lipids within their cytoplasm and eventually progress to the arterial foam cells, a hallmark of the atherosclerotic plaques. These foam cells along with the T cells constitute the fatty streak known as the earliest form of atherosclerotic lesions. The continued inflammatory response and accumulation of lipids collaborate with other events to promote atherosclerotic plaque growth and eventually rupture. During the growth phase, medial SMCs migrate to the top of the intima where they multiply and produce extracellular matrix components (fig. 1b). The SMCs and matrix molecules coalesce to form a strong fibrous cap that covers the original atherosclerotic site. Although this adds to the size of the plaque, it also seals the plaque off safely from the blood and reduces the chance of rupture. As this cap matures, some of the foam cells underneath die and lipids are released, thus forming the necrotic or lipid core of the advanced atherosclerotic lesion (fig. 1c). Eventually, the fibrous cap of a plaque might rupture, triggering thrombus formation at the site of the lesion, a process that might lead to myocardial infarction. Plaques that are most likely to break possess a thinned cap, a large lipid pool and many macrophages [4]. This plaque phenotype is partially dependent on the activities of macrophages. Macrophage foam cells secrete proinflammatory cytokines that amplify the local inflammatory response in the lesion as well as reactive oxygen species that further induce macrophage proliferation and lipid uptake. In addition, the activated macrophages produce matrix metalloproteinases that can degrade the extracellular matrix thus weakening the plaque's fibrous cap.

Ischemic heart disease is a consequence of coronary atherosclerosis. In addition to coronary bypass surgery, a common and often successful treatment is percutaneous transluminal coronary angioplasty, expanding the internal lumen

fibrous cap display decreased Cx43 expression levels and connexin expression can be no longer detected in ECs covering the fibrous cap. Beneath the lipid core, medial SMCs express both Cx43 and Cx37.

of the coronary artery with a balloon. Unfortunately, the lumen of the artery may rapidly clog again in up to 40% of the patients operated on, a process called restenosis. Therefore, stenting has recently become the preferred method for the percutaneous treatment of coronary atherosclerosis, since it has distinct advantages over angioplasty alone [5]. Indeed, stents provide a mechanical support for the treated blood vessel by expanding its diameter. In addition, the coating used on some of these devices may inhibit cellular proliferation. Thus, the possibility that neointima formation will narrow the vessel again is considerably decreased. Nevertheless, restenosis remains a major clinical problem still affecting up to 20% of patients undergoing the procedure [5]. The process of in-stent restenosis has been well described. It is essentially due to the migration of vascular SMCs from the media into the lumen of the vessel, so-called neointimal hyperplasia [6]. The first insult of the restenosis process is caused by both inflation of intracoronary balloons and the metal of the stent itself. Initially, platelets are activated and attach around the stent struts followed by the migration of inflammatory cells to the damage site where they release various growth factors and cytokines, of which platelet-derived and fibroblast growth factor (PDGF and FGF) are the most important ones [7]. Next, the released cytokines will increase proteinase expression from the matrix metalloproteinase system that then participates in the proliferation and migration of SMCs and in matrix remodeling during the exaggerated arterial wound healing process [8].

### **Use of Mice in Atherosclerosis Research**

Definition of atherogenic mechanisms in humans is hindered by the complexity and chronicity of the disease process, combined with the inability to sequentially characterize lesions in an individual patient because of shortcomings in noninvasive detection modalities.

Therefore, there has been a dependence on animal models to define mechanistic pathways in the development of the disease. Over the last decade, the mouse has become the species most used to create models of atherosclerosis. Two most commonly used mouse models of atherosclerosis are the apolipoprotein-E-deficient (*ApoE*<sup>-/-</sup>) and the low-density-lipoprotein-receptor-deficient (*LDLR*<sup>-/-</sup>) mouse [9]. Being deficient in proteins essential for lipoprotein trafficking, both mouse models exhibit hypercholesterolemia and rapidly develop atherosclerosis throughout the arterial tree in response to a high cholesterol diet. The plaques that develop are very reproducible and display some architectural features comparable to human lesions. Both mouse models have formed the basis for an enormous number of studies identifying specific molecules critical in atherogenesis.

Due to its acute and devastating nature, rupture of atherosclerotic lesions is difficult to study directly in humans. Indeed, a good animal model would help us to understand how rupture occurs and to design and test treatments to prevent it. In recent years, there has been increasing evidence of spontaneous plaque rupture in mouse atherosclerotic lesions. Evidence includes intraplaque hemorrhage and fibrin deposition, loss of fibrous cap continuity, evidence of buried fibrous caps and thrombi extending to a necrotic core. Although these histological studies represent a major advance in our concept of plaque rupture, in most cases the rupture did not cause complete vessel occlusion and could not be detected from the animal's death but rather following an extensive search for the above-mentioned signs of plaque rupture. Consequently, to date no easy-to-use and reproducible mouse model exists that exhibits lesion rupture or erosions that precede the acute cardiovascular event in humans.

### **Gap Junctions in the Vascular Wall**

Initiation and progression of atherosclerotic lesions involve complex patterns of interaction between monocyte-derived macrophages, T lymphocytes and normal cellular elements of the arterial wall, in which cytokines, chemokines, and growth factors are known to play a critical role. Apart from these paracrine signaling mechanisms, another form of direct cell-to-cell interaction involves intercellular communication via gap junctions.

Intercellular channels present in gap junctions provide a simple method of synchronizing responses in multicellular organisms through the direct exchange of ions, small metabolites and other second messenger molecules between adjacent cells [10]. This type of intercellular signaling permits rapidly coordinated activities such as contraction of cardiac and smooth muscle and transmission of neuronal signals at electrical synapses. In addition, gap junction communication plays a role in slower physiological processes such as cell growth and development. Molecular cloning studies have demonstrated that gap junction channels are formed by members of a family of related proteins called connexins in vertebrates. There are about 20 different connexin types in the human and mouse genome [11]. Each type of gap junction channel has unique inherent gating properties or permeabilities to various molecules and ions.

Within one organ or cell type, various connexins can be expressed. Cx37, Cx40 and Cx43 are generally reported to be expressed in the vascular wall [12, 13]. The distribution of connexins within the vessel wall is, however, known to be species and vessel specific [14]. ECs and SMCs have distinctive but overlapping connexin expression patterns. In general, Cx43 expression is limited to SMCs [15], although it can be detected scarcely between ECs [16, 17]. Cx40

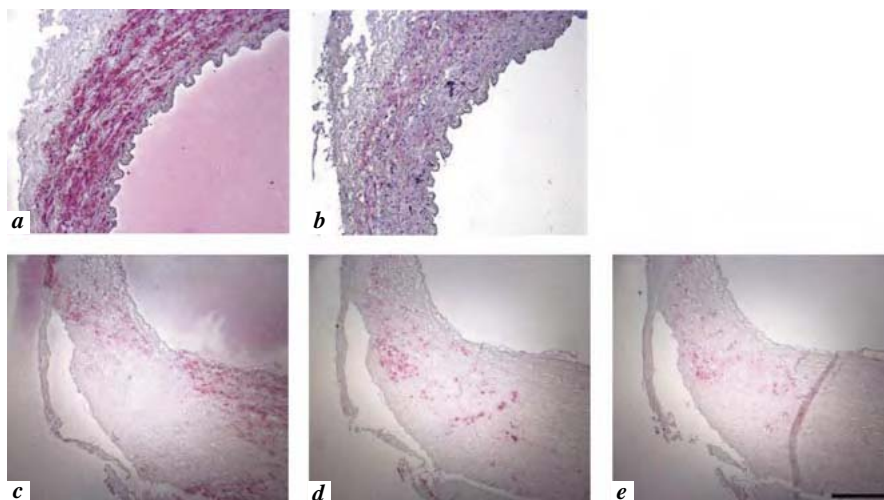
and Cx37 expression is detected in the endothelium throughout the vascular tree [17, 18], and Cx40 is observed in the SMCs of small elastic (coronary) and resistance arteries [13, 19]. Recently, additional connexins, i.e. Cx45, Cx37 and Cx31.9 have been reported to be expressed in subpopulations of vascular SMCs.

Current knowledge about vascular gap junctions is growing rapidly. These communication pathways have been implicated in a variety of vascular functions, such as coordination of vasomotor responses, regulation of angiogenesis, repair of the endothelial lining, and senescence [20, 21]. Recently, a role for gap junctions in blood vessel physiology was established in vivo. Using Cx40 knockout mice, De Wit et al. [22] demonstrated that this connexin is required for normal transmission of endothelium-dependent vasodilator responses and may be involved in the regulation of blood pressure as well. In addition, endothelial-specific deletion of Cx43 causes hypotension and bradycardia in mice [23]. The latter observation, however, remains to be confirmed as similar mice developed by another laboratory do not display a vascular phenotype [24]. Although the deletion of Cx37 leads to female infertility [25], these animals do not show an obvious vascular phenotype. In contrast to the single knock-out animals, mice that lack both Cx37 and Cx40 die perinatally and display severe abnormalities in vascular morphology [26].

### **Connexins May Be Implicated in Atherogenesis and Restenosis**

Much literature provides indirect support to the notion that connexin may play a role in the development of atherosclerosis and restenosis. First, important changes in the pattern of vascular connexin expression have been observed during atherosclerotic plaque formation and in neointima formation after balloon injury. Secondly, disturbances local fluid dynamic factors and inflammatory molecules have been shown to affect connexin expression or direct intercellular communication. Finally, changes in connexin expression or gap junction coupling have been associated with important risk factors of the disease.

In human coronary atherosclerosis, Cx43 expression in intimal SMCs is increased at early stages of the disease but reduced in advanced atheroma [27]. Hypercholesteremia-induced atherosclerosis in rabbits resulted in Cx43 expression associated with macrophage foam cells and, comparable with humans, reduced levels of Cx43 between intimal SMCs were observed in rabbit advanced lesions [28]. Similar temporal expression patterns of Cx43 in intimal SMCs and macrophage foam cells were observed during atherogenesis in *LDLR*<sup>-/-</sup> mice fed a high-cholesterol diet for 0, 6, 10 or 14 weeks [29]. Interestingly, in these mice Cx43 was expressed in the endothelium at the shoulder region of advanced



**Fig. 2.** Connexin distribution in normal human arteries and in human atherosclerotic plaques. Immunostainings on frozen sections (5 mm) of control mammary artery (*a, b*) and of an advanced atheroma in the human carotid artery (*c–e*) are shown. *a, c* Incubation with antibodies against  $\alpha$ -SM actin. *b* Incubation with Cx43 antibodies. *d* Incubation with HAM-56 antibodies. *e* Incubation with Cx37 antibodies. Note the overlapping staining patterns of  $\alpha$ -SM actin and Cx43 (*a, b*) and of the macrophage marker and Cx37 (*d, e*). Similar results were obtained in independent experiments with 3 control mammary arteries and 4 human carotid atheromas. Bar represents 60 mm in *a, b* and 200 mm in *c–e*.

atherosclerotic plaques, a localization known to experience disturbed hemodynamic forces. In addition, Cx37 was found in medial SMCs and in macrophages in the lipid core but not in the endothelium covering advanced atheromas. Also Cx40 could no longer be found in the endothelium covering these advanced plaques. These changes in connexin expression during atherosclerotic plaque development are summarized in figure 1. Similar conclusions regarding endothelial connexin expression were drawn in a later study performed by the group of Yeh et al. [30]. Indeed, they observed in C57BL/6 mice that Cx37 and Cx40 were significantly downregulated when fed the high-cholesterol diet for several months. Of note, similar expression patterns may be observed in human mammary arteries and in atherosclerotic lesions in human carotid arteries as illustrated in figure 2. Migration and proliferation of SMCs as well as synthesis of extracellular matrix by these cells are not only important players in atherogenesis, but are also key events underlying the healing response to vascular injury following balloon angioplasty, stent implantation or atherectomy [31].

Recently, a possible role for gap junction-mediated intercellular communication has been proposed in this process. Indeed, connexin expression and gap junction communication critically depend on SMC phenotype [32]. In addition, Ko et al. [33] suggested that Cx43 communication may be linked to the ability of SMCs to coordinate synthesis of extracellular matrix components, maintain the functional integrity of the elastic media and/or regulate the repair and the neointimal formation in injured vessels. Interestingly, balloon catheter injury upregulates Cx43 between SMCs in early lesions [34]. The latter observation, however, remains to be confirmed as equivalent levels of Cx43 expression in intimal and medial SMCs of the arterial wall were reported at slightly later stages after balloon injury [28]. Recently, a genetic polymorphism has been discovered in the human Cx37 protein that appeared to be a prognostic marker for atherosclerotic plaque development [35]. Furthermore, this Cx37 gene polymorphism was shown to possibly play a role in the manifestation of coronary atherosclerosis in Taiwan and Japan [36, 37].

As described above, gap junctions in arterial endothelium *in situ* are known to consist mainly of Cx40 and Cx37. However, a growing number of reports demonstrate that Cx43 is moderately expressed or absent in quiescent endothelia, but is induced under conditions associated with endothelium dysfunction. In a study examining rat aorta and its bifurcation, high levels of Cx43 in the endothelium were exclusively localized in areas facing turbulent blood flow [17]. Several *in vitro* studies also show a positive correlation between Cx43 expression and mechanical load or disturbed flow patterns [38, 39]. Taken together, these studies suggest a causal link between endothelial Cx43 expression and hemodynamic conditions that may be relevant to focal vulnerability to atherosclerosis.

There is evidence in the literature, although mainly circumstantial, for a role of gap junction communication in the inflammatory response. Altered connexin expression and/or intercellular communication have been described in a number of inflammatory conditions *in vitro* and *in vivo* [40, 41] such as the ischemic heart and after injection of endotoxin or LPS into the liver or heart. *In vitro* studies on vascular cells also show reduced gap junctional coupling with the proinflammatory mediators LPS, TNF- $\alpha$ , IL-1 $\alpha$  or IL-1 $\beta$ . Rapid closure of myoendothelial gap junctions has been reported in a coculture of human ECs and SMCs that were exposed to TNF- $\alpha$ . Interestingly, coculturing ECs with monocytes is known to increase endothelial Cx43 expression levels. Several laboratories have tested the possibility that gap junctions are involved in leukocyte transmigration *in vitro*. Thus, transmigration of leukocytes across an endothelial cell monolayer is altered in the presence of connexin-mimetic peptides or gap junction channel blockers [13, 42]. In these studies, it has been shown that inhibition of GJIC increased transendothelial migration of neutrophils,

decreased that of monocytes but had only modest effects on lymphocytes. Of major concern in the above-mentioned studies is the specificity of the GJIC-blocking reagents: pharmaceutical agents are plainly unspecific and the specificity of most of the mimetic peptides remains to be proven. Clearly, further investigation is required before definitive proof demonstrates that gap junctions do play a role in leukocyte extravasation.

Hypertension and hypercholesteremia are important risk factors for atherosclerosis. Increased Cx43 expression and gap junction communication has been described in aortas from hypertensive rats [43, 44]. Exposing cell cultures to LDL or cholesterol resulted in increased gap junction channel assembly or protection against heptanol-induced closure of Cx43-built gap junction channels [45, 46].

Altogether, these studies have stimulated our research on how vascular connexins participate in atherosclerosis, especially using the LDLR- or ApoE-deficient mouse models.

### **Connexins Are Implicated in Atherogenesis and Restenosis**

To elucidate whether the changes in connexin expression actually participate in the development of atherosclerotic plaques *in vivo*, we have intercrossed atherosclerosis-susceptible *LDLR*<sup>-/-</sup> mice with mice heterozygous for a Cx43 null mutation (*Cx43*<sup>+/-</sup> mice). As already described, the Cx43 knock-out mutation (*Cx43*<sup>-/-</sup>) in mice is lethal [47]. However, in *Cx43*<sup>+/-</sup> mice the amount of Cx43 is reduced by half [48]. Male *LDLR*<sup>-/-</sup> mice with normal (*Cx43*<sup>+/+</sup>*LDLR*<sup>-/-</sup>) or reduced (*Cx43*<sup>+/-</sup>*LDLR*<sup>-/-</sup>) levels of Cx43 of 10-week-old mice were fed a cholesterol-rich diet for 14 weeks. In these animals, lesion development was reduced by about 50% both at the level of the aortic roots and in the descending aortas compared with *Cx43*<sup>+/+</sup>*LDLR*<sup>-/-</sup> littermate controls [49]. Importantly, both groups of mice showed similar increases in serum cholesterol and triglycerides as well as body weight. One mechanism that may be responsible for decreased lesion progression in *Cx43*<sup>+/-</sup>*LDLR*<sup>-/-</sup> mice is inhibition of leukocyte infiltration into the lesions. Indeed, despite similar leukocyte counts in peripheral blood we observed almost 2-fold fewer inflammatory cells in the lesions of *Cx43*<sup>+/-</sup>*LDLR*<sup>-/-</sup> mice compared to *Cx43*<sup>+/+</sup>*LDLR*<sup>-/-</sup> controls. This suggests that Cx43-mediated gap junction communication between ECs and leukocytes may promote leukocyte extravasation, and that (partial) prevention of induction of endothelial Cx43 at the shoulder region of the lesion limits adherence/transmigration of inflammatory cells. Alternatively, Cx43 expression may affect the proliferation rate and/or apoptosis of macrophage foam cells within the atheroma. Our results also showed that the composition of atherosclerotic plaques



in *Cx43*<sup>+/-</sup>*LDLR*<sup>-/-</sup> mice was strikingly different from *Cx43*<sup>+/+</sup>*LDLR*<sup>-/-</sup> mice. Lesions of *Cx43*<sup>+/-</sup>*LDLR*<sup>-/-</sup> mice had smaller lipid-cores and fewer macrophages. In addition, the lesions had thicker fibrous caps containing more SMC and interstitial collagen. The content of SMCs versus macrophages, the extent of collagen within the lesion, and the size of the lipid core determine the vulnerability of human atherosclerotic lesions to rupture. Thus, reducing Cx43 levels in mice may favor potential plaque stabilizing processes rather than affecting plaque size alone. Altogether, these studies have provided evidence that Cx43-mediated intercellular communication plays a key role in atherosclerotic plaque formation and in the process of plaque rupture. The scenario by which reduced Cx43 ultimately leads to this dual benefit remains to be identified. Possible mechanisms involve Cx43-mediated effects on endothelium dysfunction, leukocyte migration or proliferation on the one hand as well as Cx43-mediated effects on SMC function on the other hand [50].

These findings have stirred more interest on connexin in atherosclerosis and restenosis. Our research presently focuses on investigating Cx37 and Cx40 in this disease by using atherosclerosis-prone mouse models. Preliminary data indicate enhanced atherosclerotic plaque development in *Cx37*<sup>-/-</sup>*ApoE*<sup>-/-</sup> mice [51].

The implication of Cx43 in neointimal development following balloon angioplasty in the rat carotid artery has first been reported by Yeh et al. [34]. These authors hypothesized that increased expression of Cx43 between SMCs was correlated with SMC activation and intimal growth. To determine whether altered Cx43 expression is causally related to neointimal development, we recently performed balloon distension injury in the nondissected proximal portion of the left common carotid artery of adult hypercholesterolemic *Cx43*<sup>+/-</sup>*LDLR*<sup>-/-</sup> mice in *Cx43*<sup>+/+</sup>*LDLR*<sup>-/-</sup> littermate controls. Although intimal hyperplasia could be observed on both genotypes 14 days after balloon distension, our preliminary data seem to indicate that the remaining lumen of the carotids was significantly larger in *Cx43*<sup>+/-</sup>*LDLR*<sup>-/-</sup> mice than in *Cx43*<sup>+/+</sup>*LDLR*<sup>-/-</sup> control mice [52]. Although we still have a long way to go in investigating the mechanism behind this beneficial effect, these findings may open novel therapeutic strategies for reducing neointimal disease by decreasing gap junctional communication.

## Concluding Remarks

From the studies on defined animal models discussed above, it is now established that remodeling of vascular gap junctions – notably both alterations in the level of expression of a certain connexin as well as switches in the type of connexin expressed – does occur during atherosclerotic lesion development and in the process of restenosis after balloon injury. These animal studies have been

confirmed – at least for certain stages of these disease processes – in human vascular lesions. Evidence is slowly emerging that – similar to certain paracrine cell-to-cell signaling molecules – interference with connexin expression does change the course of the diseases. For example, reducing Cx43 expression levels seems to have beneficial actions on atherogenesis as well as the process of restenosis. The mechanism and even the vascular cell types involved may, however, not be the same in both processes. Further investigations have to be done to obtain more insight in the role of gap-junction-mediated intercellular communication in vascular remodeling using mice in which connexins are deleted or over-expressed in one vascular cell type only. In addition, defined primary cell culture models may help to acquire mechanistic insight. Hopefully, such studies may identify connexin as a target for experimental interventions aimed at reducing atherosclerosis and the complication of restenosis after its treatment.

## Acknowledgements

Due to limitation in the total number of references allowed, this chapter provides only a sampling of the literature that is available on this topic and we apologize to authors whose original works have not been cited. Our work is supported by grants from the Swiss National Science Foundation (PPOOA-68883 and 3100-067777) and the Foundation Carlos et Elsie De Reuter.

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## **Connexin-Dependent Communication within the Vascular Wall: Contribution to the Control of Arteriolar Diameter**

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### **Abstract**

Communication between cells is important to the microcirculation and enables the coordination of cellular behavior along the length of the vessel. Arterioles span considerable distances within the microcirculatory network, and thus flow changes require the adaptation of vessel resistance over the whole length of the vessel in order to be effective. Such a task requires communication along the vessel wall, and gap junction channels that connect endothelial as well as smooth muscle cells with each other set the stage for this requirement. Communication along the vessel wall can be tested experimentally by confined local stimulation of arterioles either in vitro or in vivo. Certain vascular stimuli induce both a local response and a concomitant uniform remote response, confirming the rapid conduction of vasomotor stimuli along the vessel wall. Gap junctions in vascular tissue are composed of connexins (Cx) Cx40, Cx43, Cx37 and Cx45. Of these, Cx40 is of special importance: its lack results in a deficient conduction of vasodilator stimuli along the vessel wall. Interestingly, Cx40-deficient mice display an elevated mean arterial pressure, suggesting that Cx40-depending gap junctional coupling is necessary to regulate vascular behavior and peripheral resistance. While the role of other connexins is less well established, an abundance of experimental data has proven the necessity of gap junctional communication to coordinate vascular behavior during adaptive blood flow regulation.

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### **Necessity of Coordinated Cellular Responses for Drastic Flow Changes**

Endothelial and smooth muscle cells interact to control the diameters of arteries and arterioles. Within the microcirculatory network, the arterioles span considerable distances and therefore the behavior of a tremendous amount of

cells has to be orchestrated to allow synchronous diameter changes over the entire length of the vessel. The latter is a prerequisite for rapid and possibly drastic changes of blood flow in order to meet tissue oxygen demands. If dilation was limited to those parts of the vessel that are spatially closely related to the tissue, upstream resistance would become flow limiting and prevent high blood flow and sufficient oxygen delivery. This is, however, not the case and flow can increase drastically (up to 25-fold in skeletal muscle), highlighting the effective coordination of dilation. This phenomenon is called ascending dilation; it encompasses a distance of several millimeters in the microcirculation and may also affect the diameters of nonskeletal muscle vessels such as conducting vessels. In addition, large flow changes might require the coordination of vessels that are arranged in parallel. These functional requirements can only be met if cellular behavior is orchestrated in such a way that signals generated either by tissue demand (feedback control) and/or by skeletal muscle innervation (feedforward control) are communicated along the vessel wall to enable minute changes in vascular diameter along the vessel just sufficient to serve tissue needs. One coordinating signal is the physical force generated simply by the flowing blood acting on the endothelial cell layer and eliciting an upstream, flow-induced dilation [1]. Other communication pathways may contribute to achieve these requirements: Gap junctional cell coupling is certainly a candidate, as neighboring cells are connected by this means to act as a functional unit [2–4]. In addition to longitudinal signaling, endothelial and smooth muscle cells also communicate between each other. It is well established that for this transversal short-distance pathway, signaling molecules (e.g. prostaglandins or nitric oxide; NO) are involved that diffuse through the interstitial space between endothelium and smooth muscle cells. However, numerous reports have lately been published proposing that gap junctions also connect the endothelial and smooth muscle cells via myoendothelial junctions, which provide a pathway for electrical communication between the two cell layers. Aside from the transfer of electric charge, these channels could also allow the direct diffusional exchange of molecules (e.g. cAMP, IP<sub>3</sub>, Ca<sup>2+</sup>) without the dilutional effect of a transfer through the extracellular space.

### **Transversal and Longitudinal Coupling in Vessels**

Signaling via gap junctions is in principle possible along the vessel (longitudinally) or from one cell layer to the neighboring cell layer (transversally). The first communication pathway requires homocellular coupling (i.e. within a single cell layer), whereas in the latter case heterocellular gap junctions (i.e. between cell layers) are necessary. There is evidence for both types of

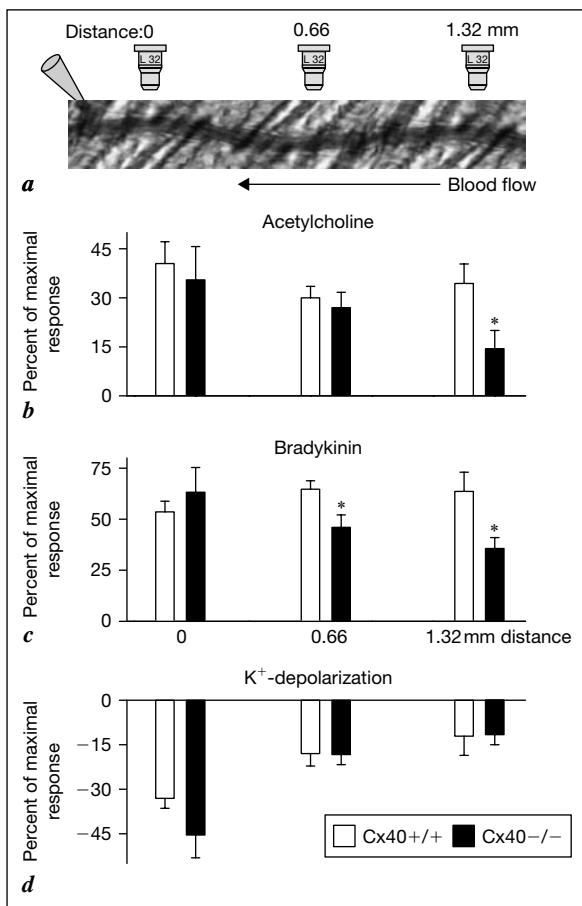
communication. In this chapter, we will discuss longitudinal cell coupling, but transversal communication via gap junctions might also be functionally important and contribute to vascular regulation. It is well established that signaling molecules (NO, prostaglandins, endothelin) can be transferred from the endothelium to the smooth muscle cells without the need for gap junctions. However, it has always been proposed that the endothelium might communicate through gap junctions to smooth muscle cells via myoendothelial junctions. The discussion whether an endothelium-derived hyperpolarizing factor (EDHF) is indeed a factor or whether the endothelium solely transfers charge to the smooth muscle via myoendothelial junctions is still ongoing. The latter would allow the transmission of endothelial hyperpolarization to the smooth muscle cell layer without the need for an EDHF. There is good experimental evidence for both hypotheses, and it is beyond the scope of this review to discuss these issues. Excellent reviews on this topic can be found elsewhere [5–7]. Moreover, myoendothelial junctions may also provide a signaling pathway for a ‘reverse’ communication, i.e. from the smooth muscle cells to endothelial cells, as shown, for instance, by studying  $\text{Ca}^{2+}$  signaling within the vascular wall. Duling and coworkers, for example, showed that  $\text{Ca}^{2+}$  originating from smooth muscle cells after stimulation with phenylephrine results in endothelial  $\text{Ca}^{2+}$  increases giving rise to a counteracting endothelium-dependent dilation via generation of NO and/or a hyperpolarization [8, 9]. The field of myoendothelial coupling is thus evolving and might lead to a better understanding of local vasomotor responses as they result from the interaction of endothelial and smooth muscle cells.

### **Conducted Vasomotor Responses Reflect Longitudinal Communication Along the Vessel**

Longitudinal signaling requires cell-to-cell coupling within at least one of the two cell layers to transmit signals along the vessel wall. As early as 1920, Krogh [10] observed that discrete stimulation within the vascular network initiated responses that spread through multiple branches, and the transmission of the ascending signal was initially attributed to local axon reflexes. The concept of ascending dilation was further elucidated by Schretzenmayr [11], Fleisch [12], and Hilton [13], who was the first to attribute the response to a conducting mechanism in the wall of the artery, specifically smooth muscle cells within the media, and proposed that the conducting mechanism contributes to hyperemic blood flow increases, as outlined above. These hypotheses were further developed by Duling and Segal, who established that different substances induce remote (also termed ‘conducted’) responses using intravital microcirculatory

approaches [14–16]. Vasomotor responses at distant sites (conducted responses) are studied experimentally by discrete stimulation of arterioles either in vitro or in vivo and assessing the response along the length of the vessel. Doing so, vasomotor responses (dilation or constriction) can be observed both at the site of stimulation and at remote sites [15, 17–20]. Usually, the remote observation points are located at upstream sites (in vivo) or the flow of the superfusate is directed towards the stimulation site away from the remote observation sites in an in vitro organ bath to exclude convective transport of the substance used for stimulation to the observation sites. Conducted responses have been observed in skeletal muscle of hamsters and mice (cremaster muscle, cheek pouch, retractor muscle) [17, 21–25] (fig. 1), and also in mesenteric, kidney, cerebral, and coronary arterioles [20, 26–28]. However, not all substances are able to induce remote vasomotor responses, and differences between stimulator compounds are found. Whereas compounds known to be endothelium-dependent dilators, such as acetylcholine or bradykinin, elicit conducted dilation (fig. 1), substances that act directly on smooth muscle by releasing NO (e.g. sodium nitroprusside) are not able to initiate remote dilation, although local dilation is of a comparable magnitude [29]. This observation shows that dilation itself does not generate the conducting signal; it also implies that flow increases that occur in response to local dilation are not responsible for the initiation of conducted dilation. Furthermore, we and others have measured wall shear stress at remote locations [25, 30], which were demonstrated not to be enhanced at the remote sites excluding a contribution of ‘flow-induced’ dilation. In addition, the time lag for flow-induced responses to develop fully is far greater (30–150 s) [31] than the time frame observed for conducted responses to reach their maximal amplitude (less than 1 s). This nearly instantaneous response also implies that fast signaling mechanisms other than diffusion are involved. Nerve conduction could meet these transmission velocities, but in the presence of blockers of fast Na<sup>+</sup> channels (tetrodotoxin; TTX), conducted responses remained intact, thus excluding the involvement of transmission via TTX-sensitive nerves [17, 20]. It should be noted that very recently evidence has been presented that TTX-insensitive sensory nerves may play a role in conducted responses at least in some preparations [32]. However, the evidence for this is scarce, and it is generally accepted that a mechanism attributable to the vascular wall itself underlies conducted dilation. Not only dilation is conducted along the vascular wall, but also locally induced constriction spread to remote sites. Local application of potassium ions or norepinephrine induces this type of response [17, 33, 34]. However, the latter does not induce a conducted response in all preparations despite local constriction similar to that found after potassium depolarization. For example, in the cremaster microcirculation of mice, norepinephrine does not induce a conducted response, whereas potassium





**Fig. 1.** Locally induced vasomotor responses conduct along arterioles. A locally confined stimulation of an arteriole (micropipette in **a**) within the microcirculation induces not only local, but also conducted responses at upstream sites. Acetylcholine (**b**) and bradykinin (**c**) induce dilations that spread along the vascular wall. The dilations at remote sites (as shown for distances of 0.66 and 1.32 mm) are always studied upstream to exclude convective transport of the stimulatory substance. Depicted are the maximal responses which occur within seconds after stimulation and appear at upstream sites with a delay of less than a second (not measurable in terms of diameter changes). Whereas in wild-type mice (white bars) the amplitude does not change within this distance, significantly smaller amplitudes are found in Cx40-deficient mice at remote sites (black bars, \* $p < 0.05$  vs. wt). Locally induced constrictions by applying a high K<sup>+</sup> solution also propagate along the vascular wall (**d**). However, amplitudes substantially diminish with distance, and no differences were found between wild-type and Cx40-deficient mice.

application does [23, 24]. In other tissues (cheek pouch of the hamster) norepinephrine is able to do so [35]. This demonstrates that vasoconstrictors must also meet certain requirements to induce a conducted response [36] and obviously this is tissue and/or species dependent.

### **What Initiates Conducted Vasomotor Responses?**

As outlined before, there are differences in the capability of dilators to initiate a conducted response. Those vasodilators known to induce remote dilation (acetylcholine, bradykinin) are endothelium-dependent dilators, and the mechanical response involves the release of prostaglandins and NO. However, the blockade of the synthesis of NO or prostaglandins did not affect the remote dilation induced by acetylcholine in the cremaster muscle [25]. Similar observations were made in other tissues: blockade of NO synthase had only a marginal effect on conducted dilation in hamster cheek pouch [29, 37]. In addition to NO and prostaglandin release, hyperpolarization is initiated, presumably by EDHF. In fact, in the presence of a high potassium solution (to abrogate any hyperpolarization due to the activation of  $K^+$  channels) or after blockade of EDHF, local and conducted responses to acetylcholine were abolished [23, 25]. Considering hyperpolarization and the high traveling speed of conduction to be necessary, it is most likely that the local change of the membrane potential spreads rapidly along the vascular wall initiating conducted dilation. In a number of studies, it has been verified that changes of the membrane potential travel along the vascular wall since hyperpolarization was detected at remote sites following local stimulation *in vitro* [33, 38–40] and *in vivo* [41, 42]. Taking it a step further, we tried to outline the site at which EDHF activity is necessary by blocking its possible targets,  $Ca^{2+}$ -dependent  $K^+$  channels ( $K_{Ca}$ ), at either the site of stimulation (local) or in a subset of experiments at remote sites. We were able to demonstrate that the activation of  $K_{Ca}$  channels is required at the stimulation site to initiate a conducted response since blockade of  $K_{Ca}$  channels at the acetylcholine stimulation site abolished local and remote responses. However, their activation was not necessary to induce remote dilation as the blockade of these channels at the remote sites did not interfere with local or conducted dilation [25]. In fact, electrical stimulation of the vessel with a hyperpolarizing current at a single site was able to induce conducted dilation on its own [43]. Taken together, these experiments suggest that locally initiated hyperpolarization (by acetylcholine or by other means) spreads along the vascular wall to induce remote conducted dilation. Important mediators of this response are  $Ca^{2+}$ -[44] and  $Ca^{2+}$ -dependent  $K^+$  channels [45], most likely mainly at the local site [25, 40, 44].

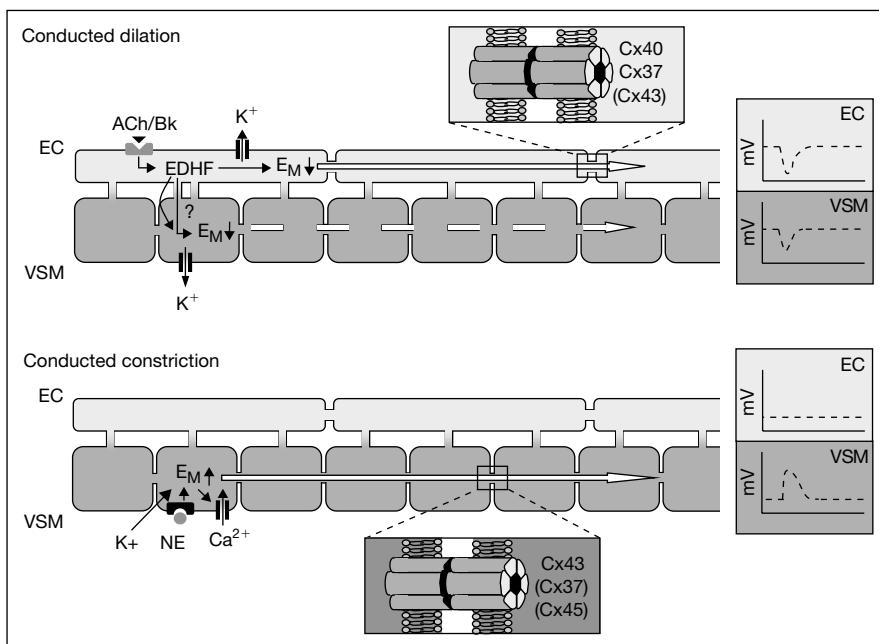
Very recently it was proposed that other mechanisms might contribute to conducted dilation. Budel et al. [34] demonstrated that the mechanisms underlying conducted dilation vary depending on the stimulus used to initiate the response. In the hamster cheek pouch preparation, bradykinin induced conducted dilation in vivo that was abrogated after inhibition of NO synthase, whereas acetylcholine was still able to initiate such a spread of dilation as outlined above. The response to bradykinin was re-established after supplementation with *L*-arginine, the precursor of NO. The authors concluded that bradykinin initiated a conducting wave that is able to release NO along the vessel wall and it is this NO release along the vessel that initiates the local and the remote response. In contrast, acetylcholine was able to use both pathways, i.e. the conduction of hyperpolarization as well as a spreading wave of NO release. However, we were also able to obtain conducted vasodilation in response to bradykinin in mice deficient in the endothelial NO synthase. Although this is in contrast to the data obtained by Budel et al. [34], we also find an attenuation of bradykinin-induced dilation after inhibition of NO synthase in wild-type mice and thus an NO dependent component, which was not found if responses were initiated with acetylcholine [25]. Therefore it has to be kept in mind that it matters which stimuli are used to initiate the response, and interpretation of the data should be done carefully.

Furthermore, conducted vasoconstriction seems to rely solely on the initiation of depolarization [33, 36]. This is obvious for the most common stimulus used, namely the local application of potassium ions onto the vessel wall, as, according to the Nernst equation, this initiates strong local depolarization. This depolarization spreads along the vessel wall inducing remote depolarization and concomitant vasoconstriction [33, 34, 36] (fig. 1). The addition of vasoconstrictors, e.g. norepinephrine, might likewise induce depolarization (depending on species and tissue) which, if sufficient in amplitude, gives rise to vasomotor responses at local and remote sites [36, 46]. Only in some preparations smooth muscle cells do not seem to be coupled very well (retractor muscle of the hamster), and in these preparations conducted vasoconstriction is not found upon stimulation with norepinephrine or potassium solution [30]. However, this seems to be an exception and might relate to a dense innervation originating from the sympathetic nerve system. This innervation pattern leaves a tight gap junctional coupling to coordinate constrictions unnecessary from a functional point of view. Most of the time, locally initiated depolarization results in local and conducted vasoconstriction and vice versa, locally initiated hyperpolarization leads to local and conducted vasodilation. Although these signals are opposite in polarity, they both reflect conduction along the vascular wall which is possible because the cells are interconnected by low-resistance channels, gap junctions.

From a functional point of view, it is important to know how far a conducted vasomotor response can travel along the arterioles, i.e. how far are the signals transmitted? An electrical signal can spread in a purely electrotonic fashion or might be regenerated by an amplifier. For a signal that spreads purely electrotonically, a length constant can be calculated since the signal dissipates with distance. This length constant is usually given as the distance at which the signal has decreased to 37% of its initial value and depends on intercellular resistance (i.e. gap junctional conductance) and on the loss of charge via the cell membrane (short-circuit current). The length constant and thus the distance covered by an electrotonic signal will be greater with lower intercellular resistance and higher cell membrane resistance. From data obtained *in vitro*, a length constant of 0.7 mm was calculated for changes in membrane potential. However, potential and diameter changes *in vivo* were found to be only slightly attenuated at distances exceeding 1 mm, especially in the case of hyperpolarization and concomitant dilation [24, 41]. The great distances where the changes in diameter can be observed suggest that a regenerative, amplifying mechanism contributes to conducted vasomotor responses *in vivo*, especially in the case of vasodilation. Similarly to the conduction process in nerves, to achieve this, the initial signal must be regenerated, with opposite polarity, however. This regenerative process may consist of voltage-gated or inward rectifier  $K^+$  channels, which can amplify the initial small hyperpolarization, enhance the local change in membrane potential and thus the concomitant diameter change. In fact, in pig coronary arterioles, blockade of inward rectifier  $K^+$  channels with barium attenuated remote dilation upon adenosine application, suggesting that the distance encompassed by the signal was related to signal amplification by these  $K^+$  channels [28].

### **Pathway for the Conducted Signal**

The fact that the vasomotor responses spread rapidly along the vascular wall and the obvious necessity of membrane potential changes suggest that the communication pathway relies on intercellular channels (gap junctions) which allow the spread of electrical charge. Gap junctions are found in both cell layers and interconnect the endothelial and the smooth muscle cells [47], and it is not obvious which cell layer transmits the signal. However, endothelial cells seem to be especially capable of transmitting the signal along the wall because of their anatomical shape and the distance one endothelial cell spans along the vessel length [48]. To find out which cell layer transmits the signal along the vessel wall, Emerson and Segal [41] developed an elegant approach to destroy the endothelial or the smooth muscle cell layer separately along the conducting



**Fig. 2.** Schematic model of signals that induce conducted vasomotor responses. Endothelial (EC) and vascular smooth muscle (VSM) cells are connected via gap junctions and hence form functional units that enable the spread of locally induced changes in membrane potential. Upper panel: conducted dilations can be initiated by stimulation of the endothelium with acetylcholine or bradykinin (ACh, Bk), which induce a hyperpolarization by the activation of  $K^+$  channels. This hyperpolarization propagates along the endothelial cell layer via gap junctions, which are composed mainly of Cx40 and Cx37 and in some tissues of Cx43. The hyperpolarization is also transmitted to smooth muscles via EDHF or direct current transfer; however, the exact nature of the signal being transferred is still unclear (indicated by ?). In some vessels, the hyperpolarization may in addition to the endothelium also spread along the smooth muscle layer. Both processes lead to remote hyperpolarization and dilation. Bottom panel: Conversely, a depolarization can be induced by norepinephrine (NE) or high  $K^+$ -solution, resulting in activation of voltage-dependent  $Ca^{2+}$  channels and local constriction. The depolarization spreads along the arteriolar wall and induces remote responses; however, it most likely travels preferentially along the smooth muscle cells which express mostly Cx43.

pathway by a light dye technique and they re-studied conduction of vasomotor responses after selective impairment of these pathways. These experiments demonstrated that in fact the endothelial cell layer is crucial to the transmission of the dilator signal to conducted sites in the cremaster microcirculation of mice [49] and also in a hamster feeding vessel residing outside the skeletal muscle [41] (fig. 2). However, it has to be kept in mind that in these vessels, smooth

muscle coupling was largely absent [30]. And, in fact, dilations were still traversing a section of injured endothelial cells in vessels within the skeletal muscle (hamster cheek pouch), showing that in other vascular beds a gap junctionally coupled, remaining smooth muscle layer is able to transmit conducted dilation [50]. Only the additional destruction of the smooth muscle cell layer prevented the transmission of the dilator signal throughout the vessel [50] (fig. 2). Most interestingly, damaging each cell layer separately at different locations along the arteriolar wall prevented the conduction of the signal through the second site of damage demonstrating that the signal is unable to jump between the two layers when traveling along the wall [34]. The aforementioned generation of an NO wave in response to stimulation with bradykinin relied solely on the endothelial cell layer: Damaging the endothelial cell layer prevented the conduction of signals generated by bradykinin through this area without affecting the spread of a response initiated by acetylcholine, in line with the previous observation that a layer of smooth muscle is able to conduct the acetylcholine-initiated signal [34].

Whereas apparently different pathways are involved in the transmission of dilator signals, in all preparations studied so far the interference with the smooth muscle layer prevented the conduction of a locally initiated vasoconstriction throughout this damaged area [50]. This observation was independent of local application of a high potassium solution or norepinephrine to initiate the response [34]. Thus, the signal to induce conducted constriction is transmitted monotonically through the smooth muscle cell layer, whereas dilator signals are able to be transmitted, at least in some preparations, through either cell layer and rely solely on the endothelium as the layer conveying the message only under certain circumstances (tissue, stimulus) (fig. 2).

### **Which Connexins Contribute to Arteriolar Coordination?**

Gap junctions are composed of connexin proteins and about 20 different connexin types are found in the human and mouse genome [51]. In vascular tissue, mainly Cx37, Cx40 and Cx43 are found [49, 52, 53]. The expression of connexins is not specific for endothelial versus smooth muscle cells. However, the cell types have distinct patterns of expression at least in larger vessels, from which most data on the expression levels of connexins were obtained. Cx43 is the connexin predominantly expressed in smooth muscle, and its expression in endothelial cells is reported to be scarce. In the endothelium, Cx40 and Cx37 are dominantly expressed, and this pattern is found throughout the vascular tree; however, Cx37 expression is less prominent than Cx40 in the endothelium [54]. Recently, the expression of additional connexin proteins has been demonstrated in the vascular wall, e.g. Cx45, most likely in smooth muscle cells. It has to be

kept in mind that the distribution patterns might be different between species and most importantly also between vessel types [55]. Whereas the expression of different connexins is relatively easily studied in large arteries, this is more difficult in the microcirculation. Gap junctions and connexins are definitely present in the microcirculation and a few studies have addressed the expression level of different connexins in arterioles. The expression of Cx43, Cx40 and Cx37 was verified in the microcirculation by immunohistochemistry [56], although it remains to be seen at which locations or in which cells. In recent studies, the expression of connexins has been studied more thoroughly and has revealed large variation in arterioles from different tissues. For example, endothelial cells in mesenteric arterioles expressed mostly Cx40 and Cx43, whereas Cx37 was expressed to a lesser degree. Surprisingly, connexin expression was not found in smooth muscle [53]. In line with this, smooth muscle cells did not express connexins in arterioles within the skeletal muscle (mouse cremaster). In endothelial cells in this tissue, however, Cx40 and Cx37 dominated among the connexins and Cx43 expression was scarce [49]. In a feed artery (hamster retractor muscle, diameter of about 100  $\mu\text{m}$ ) Cx40, Cx37 and Cx43 were found in endothelial cells, and Cx37 was found mainly in smooth muscle [57] (fig. 2). These data demonstrate clear differences in expression levels and from this it is obvious that functional data are necessary to further clarify their functions.

However, the analysis of specific roles for certain connexins has been hampered by the fact that specific blockers have only been developed recently. These substances are small peptides (gap peptides) that interfere with the extracellular domain of connexins and thus are thought to block gap junctions with a certain specificity for different connexins. These peptides have mostly been used *in vitro* to evaluate myoendothelial gap junctional communication [6]. To date, there are no studies using these peptides to interfere with longitudinal propagation of vasomotor responses along the vascular wall. A different approach is the study of connexin-deficient mice. Whereas Cx40- and Cx37-deficient animals are viable and can be studied *in vivo*, Cx43- and Cx45-deficient animals die perinatally and therefore studies are limited to mice with a cell-specific gene disruption.

Functional data from our laboratory and other investigators have demonstrated that specific connexins serve specific functions in the microcirculation. We have shown that for intact conduction of vasodilator responses along the arteriolar wall Cx40 is required. The lack of Cx40 attenuated the conduction of dilation initiated by acetylcholine or bradykinin [24] (fig. 1). In contrast, the conduction of constriction induced by  $\text{K}^+$  depolarization remained unaffected (fig. 1). These data were recently confirmed by Figueroa et al. [58], who demonstrated an attenuated propagation of dilation (but not of a constriction) induced by

electrical stimulation in Cx40-deficient animals. These data show that Cx40 deficiency has a divergent effect on constriction or dilation propagating along the vascular wall. Together with the observation that dilation and constriction travel for different distances along the arteriolar wall, they suggest that different pathways are used for signal conduction: dilation is conducted in a Cx40-dependent manner along the endothelial cell layer whereas constriction is transmitted through the smooth muscle cell layer (fig. 2). The functional observations match the expression of connexins, as we have shown that Cx40 is mostly expressed in the endothelial cell layer within the microcirculation [24], as also seen by immunohistochemistry [49]. The lack of Cx37 has not been reported to result in a defect in propagation of conducted dilations. This is consistent with experiments examining dye coupling in connexin-deficient mice: the lack of Cx40 had a stronger impact on dye transfer than the lack of Cx37 [59]. It has to be kept in mind that other connexins may be upregulated as shown in the aortas of these animals [60]. However, others have demonstrated differential regulation in endothelium and smooth muscle under these circumstances. Whereas the loss of Cx40 resulted in an upregulation of Cx37 in smooth muscle, a severe reduction of Cx37 in the endothelium was found [59]. This concomitant endothelial down-regulation of Cx37 might be the cause for the clear defects found in conduction in response to endothelium-dependent dilators outlined above. The loss of both Cx37 and Cx40 completely prevented dye coupling in aortic endothelial cells [59]. Since animals lacking Cx40 and Cx37 die perinatally, this coupling is vital [61]. Together, these results demonstrate that endothelial coupling is indispensable. Moreover, Cx40 is functionally most important to interconnect the endothelial cell layer as a main pathway for conducted dilations.

Most interestingly, Cx40-deficient animals are hypertensive, and this can be observed in anesthetized [24] as well as in awake mice [62]. This hypertension was not caused by an alteration of NO efficacy or release and is coincident with an altered nonstimulated irregular vasomotor pattern. It consisted of spontaneously appearing confined constriction that propagated to downstream sites and eventually led to complete arteriolar occlusion and intermittent flow stop [62]. At this stage, it is not clear whether the hypertension is due to increased peripheral resistance. However, heart rate was not altered in Cx40-deficient mice, which argues against a contribution of increased cardiac output to the pressure increase. Renal mechanisms might contribute to the hypertension, as Cx40 is expressed in the renin-secreting cells in the kidney [63, 64]. To assess whether enhanced angiotensin II levels as a result of increased renin release and activity contributed to the hypertension in Cx40-deficient mice, we blocked the vasoconstriction induced by angiotensin II using candesartan, an AT<sub>1</sub> receptor antagonist. This approach revealed a continuous vasoconstrictor effect of AT<sub>1</sub> receptors since the blocker resulted in a decrease in blood pressure. However,



the difference in pressure that was observed between wild-type and Cx40-deficient mice persisted, suggesting that acute pressure effects of angiotensin II were not responsible for the observed hypertension. However, this does not exclude that imbalances in renal fluid handling due to altered renin activity might have led to volume and salt overload, thereby contributing to the hypertension. In contrast, endothelial-specific loss of Cx43 resulted in hypotension and concurrent bradycardia [65]. The reason for these observed alterations in pressure regulation were not clear: the authors reported enhanced levels of NO, but also increased plasma levels of angiotensin II. We have also studied endothelial-specific Cx43-deficient mice that were generated using the same procedure (Cre-recombinase under the control of an endothelial promotor, TIE-2) and did not observe any alterations in blood pressure or heart rate [66]. The reason for these divergent results are still unclear and require further investigation. However, these data show that connexins are crucial for the function of arterioles, regulation of vascular tone, and may contribute to the physiological control of peripheral vascular resistance.

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## Cx40 Polymorphism in Human Atrial Fibrillation

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### Abstract

Previous studies have shown that two linked polymorphisms within regulatory regions of the gene for connexin40 (Cx40), at nucleotides –44 (G → A) and +71 (A → G) occur in about 7% of the general population. Cx40 is abundant in the atrium, and homozygosity for the linked polymorphisms combined with an SCN5A mutation appeared to be responsible for familial atrial standstill. We hypothesized that these polymorphisms are associated with the atrial electrophysiologic substrate favoring reentrant mechanisms for initiation of atrial fibrillation (AF). Reentry is promoted by spatial dispersion of refractoriness that can be expressed as a coefficient of dispersion (CD). **Methods:** CD was calculated from the standard deviation of 12 local mean fibrillatory intervals recorded at right atrial sites during induced AF in 30 patients without structural heart disease (14 sporadic AF episodes, 16 no AF history).  $CD \leq 3.0$  was considered normal. Cx40 genotypes were determined by DNA sequencing. **Results:** Mean CD in AF patients was  $5.96 \pm 0.70$  and without AF  $1.59 \pm 0.18$  ( $p < 0.001$ ). Thirteen of fourteen patients with AF had enhanced CD. Carriers of –44 AA genotype had higher CD compared with those with –44 GG genotype ( $6.37 \pm 1.21$  vs.  $2.38 \pm 0.39$ ,  $p = 0.018$ ), whereas heterozygotes showed intermediate values ( $3.95 \pm 1.38$ , NS). **Conclusion:** The rare linked Cx40 polymorphisms are associated with enhanced CD and thus with the substrate for reentry in AF.

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Atrial fibrillation (AF) is the most commonly encountered sustained arrhythmia in man. It is associated with significant morbidity including heart failure and thrombo-embolic events such as strokes. It is also associated with increased mortality.

AF may occur in structurally apparently normal hearts. Several studies have shown shortening of atrial refractoriness after long-lasting periods of atrial tachyarrhythmias, also known as electrical remodeling [1–7]. This remodeling

will support AF and cause further remodeling. In contrast, the development of AF in the early stages cannot be explained by a substrate due to remodeling. In these early stages, triggers – very often single or multiple premature complexes originating from the pulmonary veins [8] – are important. In addition, an initiating electrophysiologic substrate may enhance susceptibility to the occurrence of AF episodes. In previous studies, we found that patients with rare episodes of idiopathic AF (AF in the absence of structural heart disease) had enhanced spatial dispersion of refractoriness unrelated to electrical remodeling [9, 10]. This electrophysiologic substrate will favor reentrant mechanisms. Indeed, enhanced dispersion facilitated induction of AF by programmed electrical stimulation.

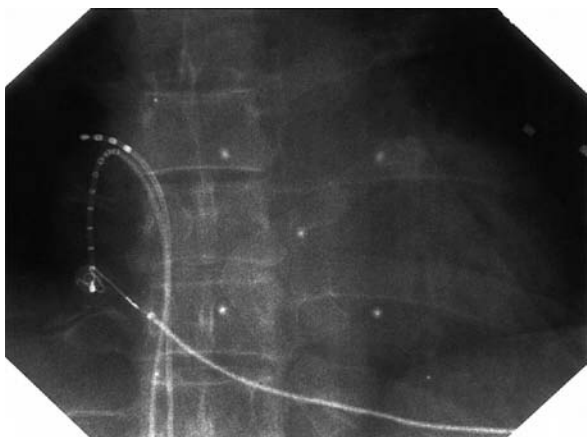
The pathophysiology and molecular mechanism of enhanced dispersion of refractoriness are still unclear. Human and animal studies have demonstrated that connexin40 (Cx40) is expressed mainly in the atrium and conduction system. Lack of Cx40 has been reported to result in increased atrial vulnerability and propensity to arrhythmias in the mouse [11]. In Cx40-deficient mice, atrial conduction was delayed and atrial arrhythmias occurred spontaneously or could frequently be induced by programmed electrical stimulation [11–13]. In addition, there is accumulating evidence suggesting that changes in expression levels and distribution pattern of Cx40 may be a cellular substrate promoting AF susceptibility and perpetuation [14].

Previous studies have shown that two linked polymorphisms within regulatory regions of the gene for Cx40 at nucleotides –44 (G → A) and +71 (A → G) occur in about 7% of the general population [15]. These linked polymorphisms combined with a mutation in the cardiac sodium channel gene SCN5A were associated with familial atrial standstill [15]. We hypothesized that the rare linked Cx40 polymorphisms are related to enhanced spatial dispersion of refractoriness and thus with susceptibility to reentry and AF.

## Methods

### *Patients*

A total of 30 patients (10 women, 20 men; age 18–52, mean 32.8 years) referred for catheter ablation because of supraventricular tachycardia (27 atrioventricular accessory pathway, of which 2 concealed, and 3 atrioventricular nodal reentrant tachycardia) participated in the study after informed consent. Fourteen had prior documented sporadic episodes of AF (AF group) and 16 had no evidence of AF (control group). Electrocardiographic documentation of  $\geq 1$  episode of AF was required for inclusion in the AF group. Patients in the control group did not have any history of irregular heartbeat. The mean number of AF episodes in the AF group was 1 (range 1–5), median duration 1 h (range 15 min to 3 h), with a mean asymptomatic interval prior to the electrophysiologic study of 148 days (range 9–365 days). Electrocardiographic telemetric monitoring was carried out for  $\geq 24$  h immediately before the electrophysiologic study. None of the patients had AF episodes during the telemetric observation period.



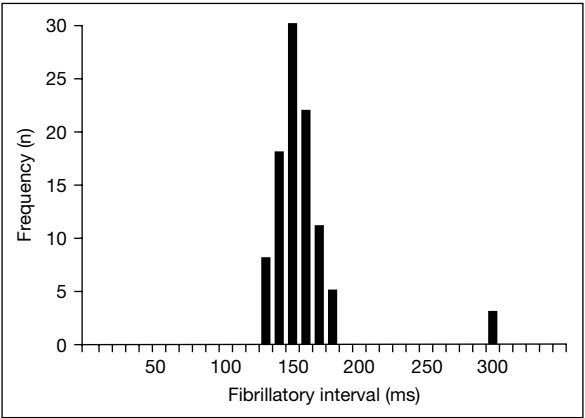
**Fig. 1.** Fluoroscopic image in anteroposterior projection showing the decapolar catheter at the right atrial lateral free wall and the quadripolar catheter in the right atrial appendage. Decapolar catheter position was consistent in all patients and was designed to maximize the number of electrodes with firm wall contact and to maintain catheter stability during the study. In addition, ventricular far-field interference in unipolar electrograms was minimized with the use of this position. From Ramanna et al. [9], with permission from the American Heart Association.

To exclude structural heart disease and conditions with a potential effect on cardiac hemodynamic or electrophysiologic functions, all patients underwent physical examination, 12-lead electrocardiography, 2D echocardiography and hematologic and biochemical testing. None of the patients were on antiarrhythmic drugs,  $\beta$ -blockers or digitalis.

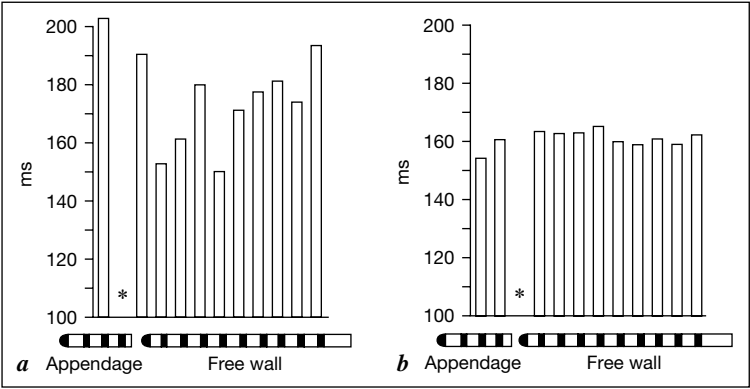
#### *Electrophysiologic Study*

All patients were studied in the fasting, nonsedated state. The protocol has been described previously [9]. Briefly, through right femoral venous access, a decapolar catheter was positioned at the right atrial free wall, as shown in figure 1, and a quadripolar catheter was placed in the right atrial appendage. Twelve unipolar electrograms were recorded, 10 from the decapolar and 2 from the quadripolar catheter. AF was induced with an increasingly aggressive stimulation protocol starting with a single unipolar extrastimulus subsequently from all electrodes, up to aggressive burst pacing with cycle lengths of 100–50 ms at 4 times the diastolic threshold current. Induction of AF episodes of at least 1 min was required.

After AF induction, the segment between 15 and 30 s after AF onset was used for electrogram analysis. Fibrillation intervals were measured at all recording sites. A histogram of mean fibrillatory intervals at a single site is shown in figure 2. The mean of all fibrillatory intervals at a single site was used as an index for the local refractory period. In figure 3, mean local fibrillatory intervals are shown in a patient with prior paroxysmal AF, and in a control patient, respectively. It is clear that the dispersion of refractoriness is increased in patients with AF. The average and SD values for these indices were calculated. Spatial dispersion of refractoriness was determined by calculating the coefficient of dispersion (CD), defined as the standard deviation of all local mean fibrillatory intervals expressed as a percentage of the overall mean fibrillatory interval. On the

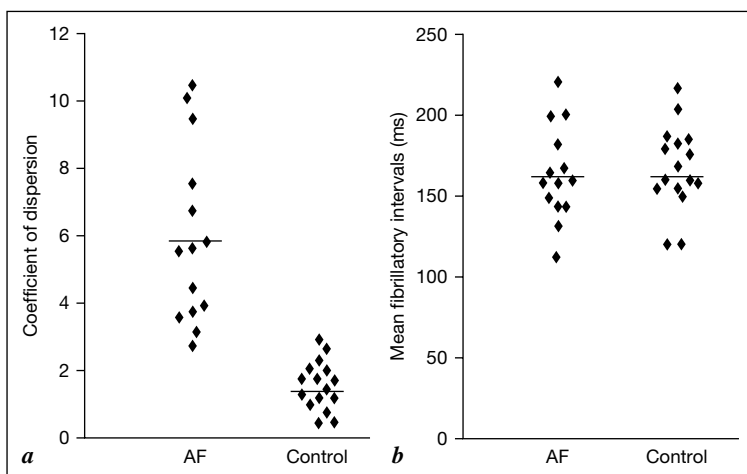


**Fig. 2.** Histogram of mean fibrillatory intervals at a single atrial recording site. Mean local fibrillatory interval was defined as average of intervals, in this case 154 ms. This mean fibrillatory interval was used as an index for local refractoriness. From Ramanna et al. [9], with permission from the American Heart Association.



**Fig. 3.** Mean fibrillatory intervals (in ms) for each measurable atrial recording site in a typical patient with prior paroxysmal AF (**a**) and in a control patient (**b**). The patient with prior AF displayed more inhomogeneous mean fibrillatory intervals than did the control patient. AF could be induced with a single extrastimulus in the patient with AF whereas in the control patient, it was induced with burst pacing only. The electrodes marked with an asterisk had inadequate signal quality and thus were excluded from analysis. From Ramanna et al [9], with permission from the American Heart Association.





**Fig. 4.** *a* CD in 14 patients in the AF group and 16 in the control group. Patients with prior AF had significantly higher CD than in the control group. *b* Mean fibrillatory intervals in both groups: there were no statistically significant differences. Mean values are indicated by horizontal lines in *a* and *b*.

basis of our previous study [9], a CD value of  $\leq 3.0$  was considered normal, whereas  $CD > 3.0$  was considered enhanced spatial dispersion of atrial refractoriness.

#### *Molecular Biology Study*

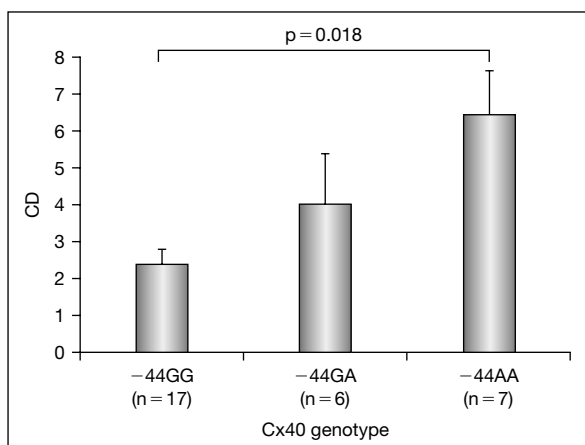
Genomic DNA was extracted according to standard protocols. Cx40 polymorphisms were detected by direct sequencing as previously described [15]. Analysis was performed blinded to the electrophysiologic findings.

#### *Statistical Analysis*

For detailed data and statistical analysis, see Firouzi et al. [16].

## **Results**

In the AF group most patients had inducible AF with only a single atrial extrastimulus, whereas burst pacing was required in only one out of 14 patients. In the control group burst pacing was required in the large majority of patients. CD values and mean fibrillatory intervals in all patients are shown in figures 4a, b, respectively. The AF and control groups are compared. Mean CD in AF patients was  $5.96 \pm 0.70$  and in control group  $1.59 \pm 0.18$ . In the AF group, 13 out of 14 patients had enhanced CD ( $> 3.0$ ), whereas in the control group all patients had a normal CD ( $\leq 3.0$ ). This difference was significant ( $p < 0.001$ ). In contrast,



**Fig. 5.** CD in the study population stratified according to the Cx40 genotype. p value was calculated by one-way ANOVA followed by Bonferroni correction. Values are mean  $\pm$  SEM. From Firouzi et al. [16], with permission from the American Heart Association.

mean fibrillatory intervals were not different, indicating absence of remodeling as substrate for AF in this study.

All patients with allele G at position -44 had allele A at +71, and vice versa. Thus, three groups could be distinguished: -44AA/+71GG, -44GG/+71AA, and the heterozygous group -44GA/+71GA. AF was induced more easily, i.e., with 1 extrastimulus in patients with the -44AA genotype than in those with the -44GG genotype (86 vs. 29%,  $p = 0.042$ ). Carriers of -44AA genotype had a significantly higher CD compared to those with -44GG genotype ( $6.37 \pm 1.21$  vs.  $2.38 \pm 0.39$ ,  $p = 0.018$ ; fig. 5) Heterozygotes had intermediate values ( $3.95 \pm 1.38$ , NS).

## Discussion

In the early stages of AF and in the absence of structural heart disease, the occurrence of AF episodes depends on triggers and an initiating electrophysiologic substrate. This substrate appeared to be related to enhanced spatial dispersion of refractoriness. Linked Cx40 polymorphisms occurring in 7% of the general population appeared to be strongly associated with this substrate. Since enhanced dispersion promotes reentrant mechanisms by creating a unidirectional block, we have to realize that only AF due to reentry will be facilitated.

Although it is less likely that a focal tachyarrhythmia will be facilitated by enhanced dispersion of refractoriness, fibrillatory conduction may be due to this dispersion. A focal tachyarrhythmia with fibrillatory conduction will mimic AF due to multiple wavelet reentry. Because of difficulties in proper definition of AF from the surface ECG, analysis of genotype-to-phenotype relationships at the level of well-defined electrophysiologic parameters, such as dispersion of refractoriness, may be more unequivocal. It is very likely that other factors such as fibrosis, conduction, load mismatch and ion-channel polymorphisms contribute to susceptibility to initiation of AF as well. However, these factors were not specifically analyzed in this study. Further research is needed to clarify the role of Cx40 polymorphisms and other factors for propensity to initiation of AF episodes.

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